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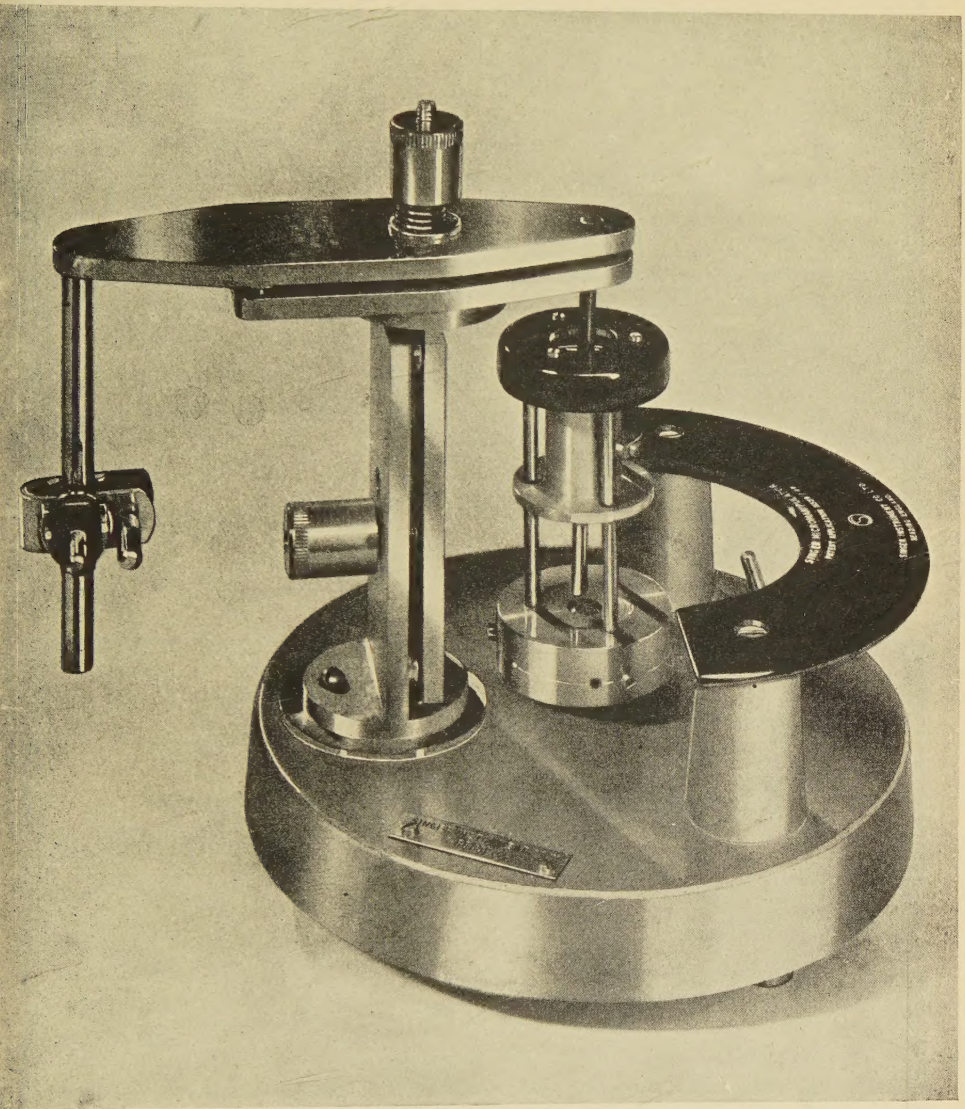

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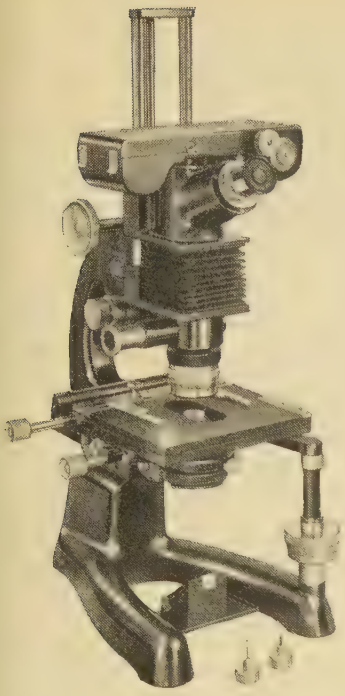

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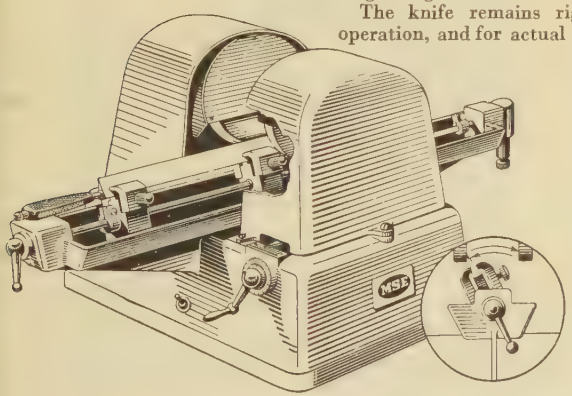

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The Structure and Function of the Basement Membrane Muscle System in *Amphiporus lactifloreus* (Nemertea)

By J. B. COWEY

(*From the Dept. of Zoology, King's College, Newcastle-upon-Tyne*)

With one plate (fig. 5)

Summary

The body wall of *A. lactifloreus* has the following structure from the outside inwards.

(i) A basement membrane of five to six layers immediately underlying the epithelium. Each layer consists of right-hand and left-hand geodesic fibres making a lattice, whose constituent parallelograms have a side length of from 5 to 6μ. The fibres are attached to one another where they cross; so there can be no slipping relative to one another.

(ii) A layer of circular muscle-fibres running round the animal containing two systems of argyrophil fibres—one of fibres at intervals of 10μ running parallel to the muscle-fibres and the other of fibres running radially through the layer from the basement membrane to the myoseptum.

(iii) A myoseptum which is identical in structure with a single layer of the basement membrane.

(iv) A layer of longitudinal muscle, whose fibres are arranged in layers on each side of a series of longitudinal radial membranes.

Membranes identical in structure with the basement membrane invest the nerve cords, the gut, the gonads, and the proboscis.

The interrelations of argyrophil and muscle-fibres in the muscle layers is described and their functioning discussed.

The system of inextensible geodesic fibres is analysed from a functional standpoint. The maximum volume enclosed by a cylindrical element (cross-section circular), of such a length that the geodesic makes one complete turn round it, varies with the value of the angle θ between the fibres and the longitudinal axis. When θ is 0° the volume is zero; it increases to a maximum when θ is 54° 44′ and decreases again to zero when θ is 90°. The length of the element under these conditions varies from zero when θ is 90° to a maximum (the length of one turn of the geodesic) when θ is 0°.

The body-volume of the worm is constant. Thus it has a maximum and minimum length when its cross-section is circular, and at any length between these values its cross-section becomes more or less elliptical. It is maximally elliptical when θ is 54° 44′, i.e. when the volume the system could contain, at circular cross-section, is maximal. From measurements of the ratio of major to minor axes of this maximally elliptical cross-section, the maximum and minimum lengths of the worm relative to the relaxed length and values of θ at maximum and minimum length are calculated. The worm is actually unable to contract till its cross-section is circular; but measurements of its cross-sectional shape at the minimum length it can attain, permit calculation of the theoretical length and value of θ for this cross-sectional shape.

Calculated values of length and the angle θ agree well with the directly observed values.

[Quarterly Journal of Microscopical Science, Vol. 93, part 1, pp. 1–15, Mar. 1952.]

THE littoral nemertines commonly met with show extraordinary powers of contractility and extensibility. It has always been assumed that inelastic collagenous material formed the skeletal tissues in these animals. Thus, it seemed probable that they had some special arrangement whereby inelastic collagen could be made to meet the requirements of the changes of shape that occur. An investigation of the microscopical structure and properties of the basement membrane and body-wall musculature has therefore been made. The structures in nemertines that are usually regarded as collagenous have a special affinity for silver; in this paper they will be called 'argyrophil' or 'argentophil'.

Material and Methods

Living *Amphiporus lactifloreus* (Johnston), supplied by the Marine Biological Stations at Cullercoats and Plymouth, were used. The animals were usually anaesthetized in magnesium chloride sea-water before fixing. This process paralyses the contractile mechanism of the muscle-fibres and the animal takes up what I shall refer to as the relaxed condition. Once in this condition it can be passively stretched and fixed at any desired length up to its maximum. Heidenhain's Susa, Bensley's formalin-Zenker, and a 4 per cent. solution of formaldehyde in sea-water were used for fixation. This was followed by Peterfi double-embedding and sectioning at 10μ, 4μ, and 2μ. Sections were stained in Mallory's triple stain, Heidenhain's Azan, and iron haematoxylin counterstained with Van Gieson, as stock methods. Silver impregnation by Wilder's reticulum method (Wilder, 1935) gave the best preparations for micro-anatomy.

The Micro-anatomy of the Argyrophil Tissues

Transverse Sections

A general view of the structure of the body-wall of *Amphiporus lactifloreus*, as revealed by the researches described in this paper, is shown in fig. 1.

In sections stained with Mallory's triple or Heidenhain's Azan stain, the argyrophil tissues take up the aniline blue very strongly. The principal argyrophil structure is the basement membrane immediately underlying the epithelium (fig. 2). It is about 5μ thick and forms a continuous cylindrical membrane round the animal. The staining is fairly even and homogeneous, there being little to suggest a fibrous structure with Mallory or Azan.

The circular muscle layer lies immediately beneath the basement membrane and aniline blue staining fibres or membranes are to be seen running parallel to the muscle-fibres at intervals of approximately 10μ. Böhmig (1930) and Burger (1897) both describe a layer of diagonal muscle-fibres lying between the circular and longitudinal muscle layers. This layer is said to be peculiarly well-developed in genera including *Amphiporus*. In *Amphiporus lactifloreus* I find it to be composed of only a few fibres very widely separated, and it must be mechanically unimportant. It is in such close contact with the myoseptum that it was found to be impossible to determine on which side of this structure

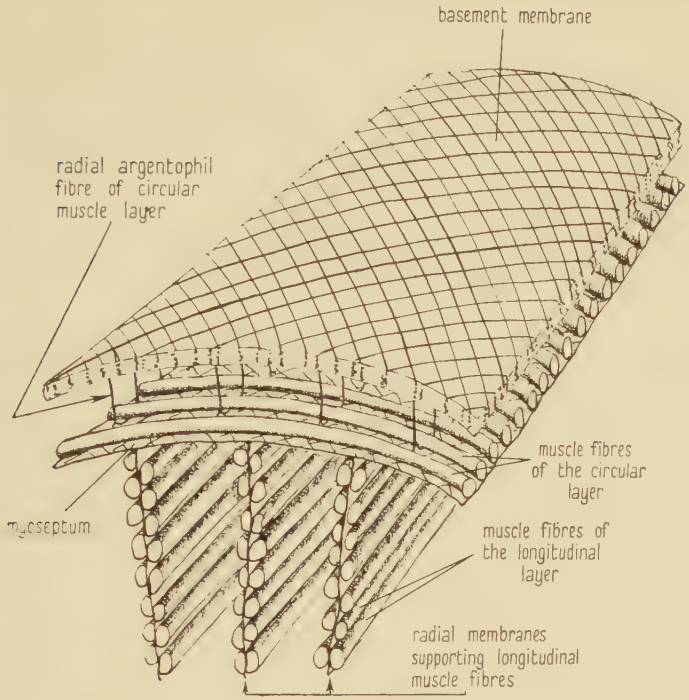

FIG. 1. Diagram summarizing the structure of the body-wall in *Amphiporus lactifloreus*. In the interests of clarity the weak diagonal musculature, which lies between the circular and longitudinal, has not been figured.

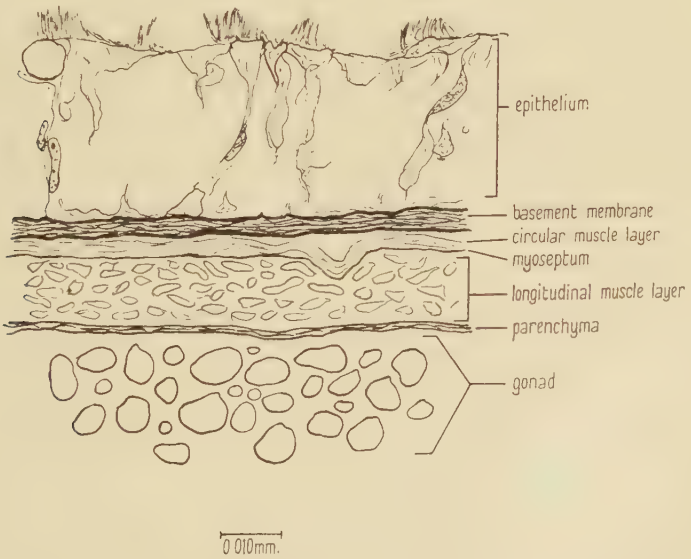

FIG. 2. Part of transverse section of a relaxed *Amphiporus lactifloreus*.

it occurs. It has not been discussed in the present paper nor has it been figured.

Between the circular and longitudinal muscle layers another argyrophil membrane occurs which stains like the basement membrane. It is only about 1μ thick: it will be called the myoseptum. The longitudinal muscle layer itself appears to have only odd strands of argyrophil tissue running through it in a roughly radial direction.

The body-wall is separated from the gut and gonads by a parenchymatous layer of varying thickness. It contains irregular argyrophil membranes and fibres.

Argyrophil membranes invest most of the internal organs of the animal. They occur, for instance, round the lateral nerve-cords, the gonads, and the gut, and form an essential part of the structure of the wall of the rhynchocoel and the proboscis itself. They differ from the basement membrane only in thickness.

Silver impregnation of a transverse section of a worm fixed in the relaxed condition (fig. 3) shows the basement membrane to be fibrous in nature. The fibres do not run round the animal in the plane of the section, for only a short length of any one fibre can be seen.

The circular muscle layer shows some radial fibres of argyrophil material running from the basement membrane to the myoseptum, which can be seen to be a single layer. The fact that the fibres of the basement membrane and myoseptum do not run in the plane of the section, but obliquely to it, is doubly plain here.

In the longitudinal muscle layer there are regularly occurring radial membranes of argyrophil material. They are spaced at intervals of 10μ and run from the myoseptum to the parenchyma. The parenchyma itself presents an appearance of intertwined, heavily stained fibres. The membranes investing other organs show a structure similar to that of the basement membrane.

In stretched worms (fig. 4) the basement membrane takes on a rather dotted appearance; this is presumably because the fibres in it are more nearly orientated in a direction perpendicular to the plane of the section. This is true of all the fibrous membranes in the animal and is particularly apparent in the case of the myoseptum.

Longitudinal Sections

Longitudinal sections, cut in the plane of the basement membrane and body-wall muscle layers and stained by a trichrome method, show very little of the detailed structure of the argyrophil material. It is possible, by extremely careful differentiation of the aniline blue, to show that the basement membrane contains a regular lattice-work of fibres which stain a rather more intense blue than the matrix in which they are set.

Silver-impregnated sections, however, give a very clear demonstration of this lattice (fig. 5, E and F). The photograph E is taken from a preparation made from a stretched worm, whilst F is from a contracted worm.

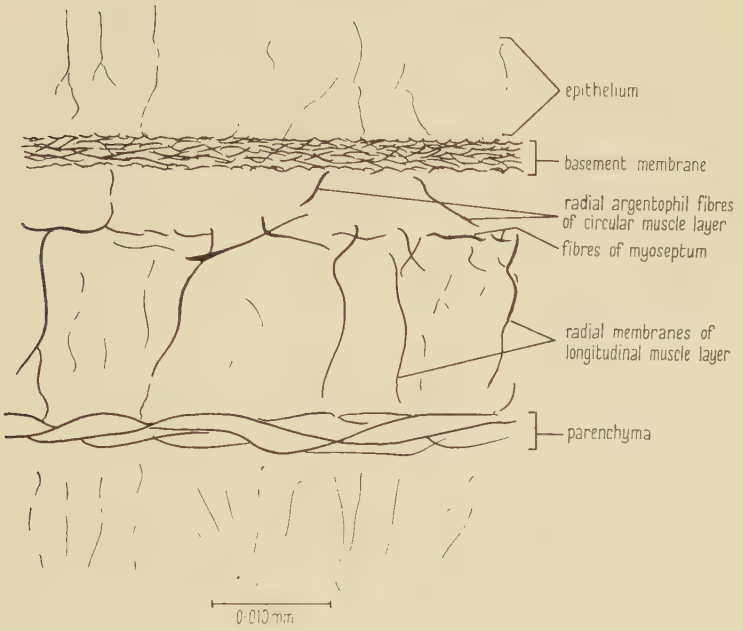

FIG. 3. Transverse section of a relaxed worm. Fixed in formalin and silver-impregnated; hence showing only argyrophil structures.

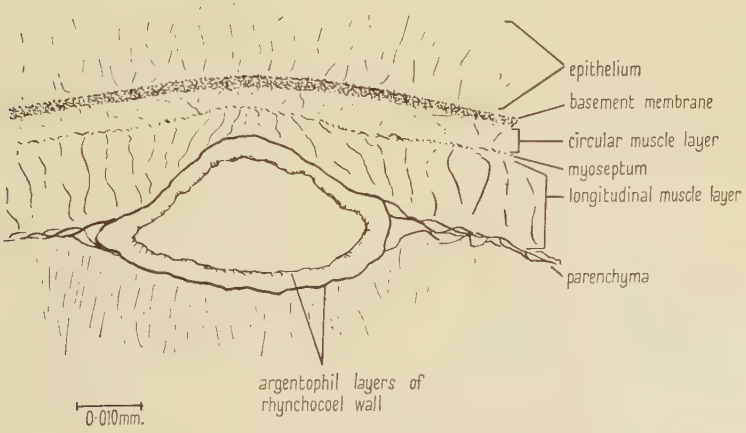

FIG. 4. Transverse section of stretched worm showing structure made apparent by silver impregnation.

The basement membrane is found to be made up of from four to six layers, and sections passing obliquely through it show the cut edge of each layer. An example of this type of arrangement can be seen in the membrane round the gonad (fig. 5 A). Examination of the individual fibres of the lattice with a 2-mm. apochromatic oil-immersion objective, showed that each layer consisted of two sets of fibres running round the animal in opposite directions at the same angle to the longitudinal axis. The parallelograms produced by this system have a side length of from 5μ to 6μ in stretched specimens. The fibres are set in a lightly staining matrix which apparently preserves their positions relative to one another. The angle between the direction of the fibres and the longitudinal axis *A–P* of the animal varies from 10° to 80° as the overall length of the animal changes in the ratio of 5 to 1 (fig. 5, E and F).

Photographs G and H (fig. 5) show areas of circular muscle in which the plane of the section has run slightly obliquely through the circular muscle layer. All three pictures show lines of silver deposition running round the animal parallel to the circular muscle-fibres, which can be identified as a faintly distinguishable haze in the background, quite distinct from the narrower and very black lines of deposition. These black lines are not merely granular deposits of silver with no underlying structure for they are clearly demonstrated by Mallory or Azan staining. The worms from which these sections were cut were all completely contracted, i.e. their circumference was at its maximum value, and one would expect any encircling fibres to be perfectly straight as these structures manifestly are. In photograph H adjacent fibres seem to be linked together by a loop which gives rise to another fibre with connexions that cannot be determined. These loops only occur in regions where circular and longitudinal muscles are in close proximity to one another. Photographs B and C (fig. 5) show other argentophil fibres in the circular muscle layer. These run radially (i.e. perpendicularly to the plane of the muscle-fibres) from the basement membrane through the circular muscle layer, and end in the junctional region between circular and longitudinal muscle layers. Most of them end in a forked structure which is probably

EXPLANATION OF PLATE

FIG. 5. Fixation in a 4 per cent. solution of formaldehyde in sea-water, Peterfi double embedding and sectioning at 4μ followed by Wilder's silver impregnation method (said to be specific for reticulin in mammals). A. Part of a longitudinal section in which the plane of section passes obliquely through the membrane investing the gonad. The layered structure of this membrane is very apparent. B and C. Longitudinal sections in which the plane of the section is oblique to the basement membrane and circular muscle layer. As a result of this the radial argyrophil fibrils of the circular muscle layer are well shown. D. Part of a longitudinal section in a plane oblique to that of the basement membrane. The diagonal muscle-fibres are clearly visible. E. Longitudinal section in the plane of the basement membrane and circular muscle layer. The fibre lattice of the basement membrane is easily distinguishable whilst that of the myoseptum can be seen showing through the circular muscle layer. The animal used for this preparation was stretched. F. The same as E except that it was made from a contracted worm. G and H. Longitudinal sections from contracted worms. The plane of section runs slightly obliquely through the circular muscle layer. Evidence of the presence of the myoseptum can be seen as well as the argyrophil fibrils of the muscle layers. H shows the loops connecting adjacent argyrophil fibrils very clearly.

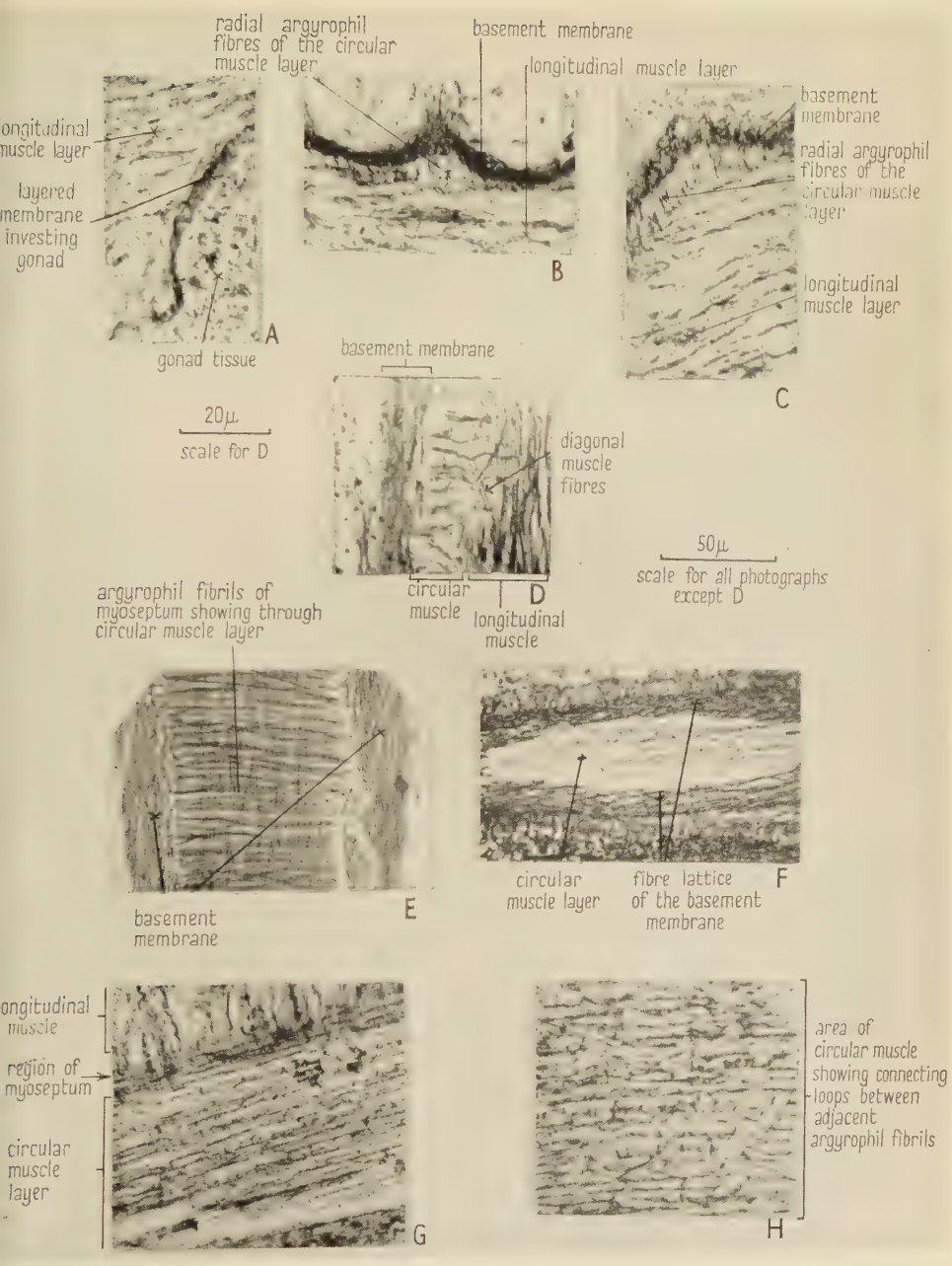

FIG 5.

J. B. COWEY

dentical with the loop described previously. Further evidence for this view s available in fig. 6, which represents part of a median longitudinal section of stretched worm. Radial fibres in the circular muscle layer which bifurcate n the region of the myoseptum, can be seen.

The single layer lattice of the membrane between the two muscle layers is ıot clearly depicted in any of the photographs. It is, however, easily visible n both silver and aniline blue stained preparations.

The longitudinal muscle layer shows argentophil fibres running parallel to he muscle-fibres. Close examination of fig. 5, B and C, where the plane of ection approached a radial direction, indicates that at least some of them are ıot, in fact, fibres but radially arranged sheets of argentophil material.

In the foregoing account the following 'collagenous' or argentophil structures have been noted:

(i) the basement membrane with its layered fibre lattice structure;
(ii) the 'circular fibres' of the circular muscle layer;
(iii) the radial fibres of the circular muscle layer running from the basement membrane and ending in a bifurcation in the myoseptum;
(iv) myoseptum with its fibre lattice;
(v) the radial membranes of the longitudinal muscle layer;
(vi) the membranes and fibres of the parenchyma;
(vii) the fibre lattice membranes investing the wall of the rhynchocoel, the proboscis, the lateral nerve-cords, and the gonads.

The Structure of the Muscle Layers

Fig. 6 shows that in stretched preparations the circular muscles form a ayer one fibre thick. This layer is penetrated at intervals of about 10μ by the adial argentophil fibres. When the worm contracts the distance between the asement membrane and the myoseptum between the muscle layers becomes reater. Hence the muscle-fibres must form a layer, two or three fibres thick, nd the radial argyrophil fibres will be much closer together (about 2μ apart). Observation confirms both these points. It is suggested that in the contracted vorm the radial fibres are so connected between the basement membrane and he myoseptum, that extension of the worm causes one end of these fibres to nove either anteriorly or posteriorly relative to the other end; i.e. extension as the effect of making the radial fibres lie down in an antero-posterior irection. Muscle-fibres attached to one side of these radial fibres would then orm a layer one fibre thick when the worm is extended and two or three ibres thick when the worm is contracted (fig. 7).

Fig. 8 shows short lengths of two adjacent circular muscle-fibres from a elatively extended worm. They are linked to one another by an argentophil ibril which is attached to them alternately along its length. Contraction of he worm will force the muscle-fibres closer to one another and at the same ime increase their length. This would result in two muscle-fibres being closely pplied to one another with an argentophil fibril between them and could give

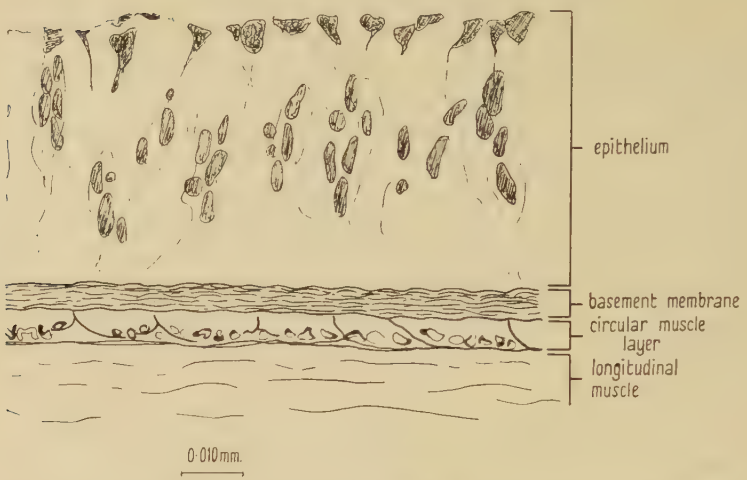

FIG. 6. Part of a longitudinal section of a stretched worm. Silver impregnation.

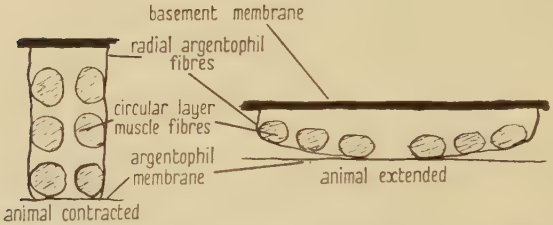

FIG. 7. Diagram attempting to explain how the muscle-fibres of the circular muscle layer are arranged in stretched and contracted animals.

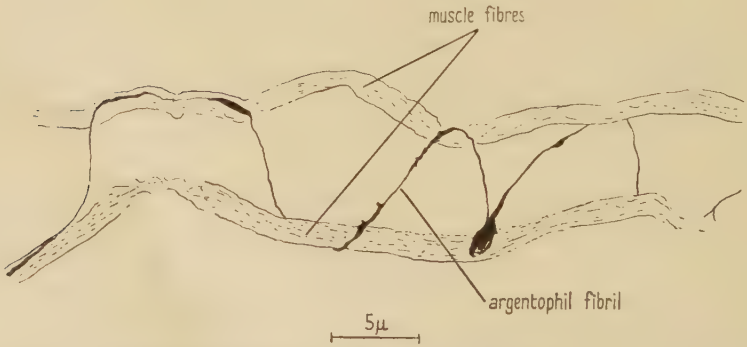

FIG. 8. Two muscle-fibres showing the argyrophil fibril linking them together.

an appearance similar to those illustrated in fig. 5, G and H. This argentophil fibre is probably one of those already mentioned which stain with aniline blue.

The longitudinal muscle-fibres are attached to the longitudinal, radial membranes (fig. 1). In the contracted condition each membrane has a single layer of muscle-fibres on each side of it. When the animal is extended, to its maximum length the radial membranes are very much shortened in a radial direction and hence have a band of muscle three or four fibres thick attached on each side.

Relaxed muscle-fibres in both layers quite frequently show a zigzagging course with a sharp change in direction every 5–6μ. The angle turned through in each change in direction suggests that they are closely attached to the lattice of the myoseptum.

The Properties of the Argentophil Membranes and Fibres

The following definitions are necessary:

(i) relaxed length is that length which the worm attains when it is completely anaesthetized by magnesium ions;

(ii) minimum length is the shortest length produced in the living worm by complete contraction of the longitudinal muscles;

(iii) maximum length is the length to which a magnesium anaesthetized worm may be passively stretched before it breaks.

The ratio of minimum length to relaxed length to maximum length was approximately 1 : 3·3 : 5·6 in some 25 specimens of *Amphiporus lactifloreus* that were examined. The relaxed length is apparently an equilibrium position in which there is no resultant force tending to change the length of the animal. This can be illustrated by taking an anaesthetized worm and stretching it to its maximum length and then releasing it. It quickly (5 seconds) returns to the relaxed length. This process can be repeated again and again.

Pieces of rat's aorta and pieces of nemertine were fixed in the same fixative, embedded, sectioned, and mounted alongside one another on the same slide. They were then stained for elastic tissue by Weigert's method and the orcein method. In both stains elastic fibres in the aorta section gave a very distinct positive result whilst the nemertine sections were completely unstained. This shows that the nemertine material is certainly not like the elastic tissue of the vertebrates from the point of view of its staining properties, but certainly does not exclude the possibility that it is extensible.

Micro-dissection of fresh nemertine material showed unequivocally that the individual fibres of the basement membrane are inelastic. They can be snapped on micro-needles but will not stretch.

The Functioning of the Fibre System

The body of a nemertine is bounded by two networks, that of the basement membrane and the myoseptum. Both these have a similar structure, which is the same from every aspect of the animal, dorsal, ventral, or lateral. They are composed of more or less inextensible fibres of collagen arranged along

right-handed and left-handed geodesic helices. The fibres are thus connected together to form a lattice. Their connexions prevent them from slipping relatively to each other. In such a system extension and contraction can only be accommodated by changing the angle which the elements of the lattice make with the longitudinal axis of the animal, just as in the extension and retraction of lazy-tongs.

At any particular length of the animal, the geodesic helices of the lattice will thus bear a particular angle to the longitudinal axis of the animal. At that length also, the system will be able to enclose a certain maximum volume. This condition will obtain when the system is a cylinder of circular cross-section. Should the volume of the enclosed worm be less than this maximum, then the cross-section of the cylinder will become elliptical; though so long as the length of the system remains constant, the length of the perimeter of the section remains unchanged, as does also the angle of the geodesic helices.

Let us determine the relation of the greatest volume of a cylindrical element of a given length, to the angle of the geodesic helices of the lattice. Consider a cylindrical element of such length that it carries one complete turn of the helices round it, as in fig. 9A. Imagine such an element to be cut along its length, parallel to the longitudinal axis and spread out flat to give the condition figured in fig. 9B.

Let l = length of the cylinder.
r = its radius.
D = the (constant) length of geodesic which makes one complete turn round the cylinder.
θ = the angle between the direction of the geodesic and the longitudinal axis of the cylinder.
v = the greatest volume of the cylindrical element at length l (that is, the volume at circular cross-section).

From fig. 9B

$$r = \frac{D \sin \theta}{2\pi}$$

$$l = D \cos \theta$$

and

$$v = \pi r^2 l,$$

Hence

$$v = \frac{D^3 \sin^2\theta \cos \theta}{4\pi}$$

and

$$\frac{4\pi}{D^3} v = \sin^2\theta \cos \theta.$$

Fig. 9C shows the relation of the greatest volume of the cylindrical element at any length to the angle θ. If the element is caused to elongate θ approaches zero and its greatest volume approaches zero also. If the element is able to shorten indefinitely θ approaches 90°, and again its greatest volume becomes

zero as the element approaches the form of a flat disk. At a certain value, when $\theta = 54^\circ 44'$, the greatest volume of the element at any length becomes maximal. If the element possessed this volume in the living worm, the worm could neither elongate nor contract, since to do either would necessitate a

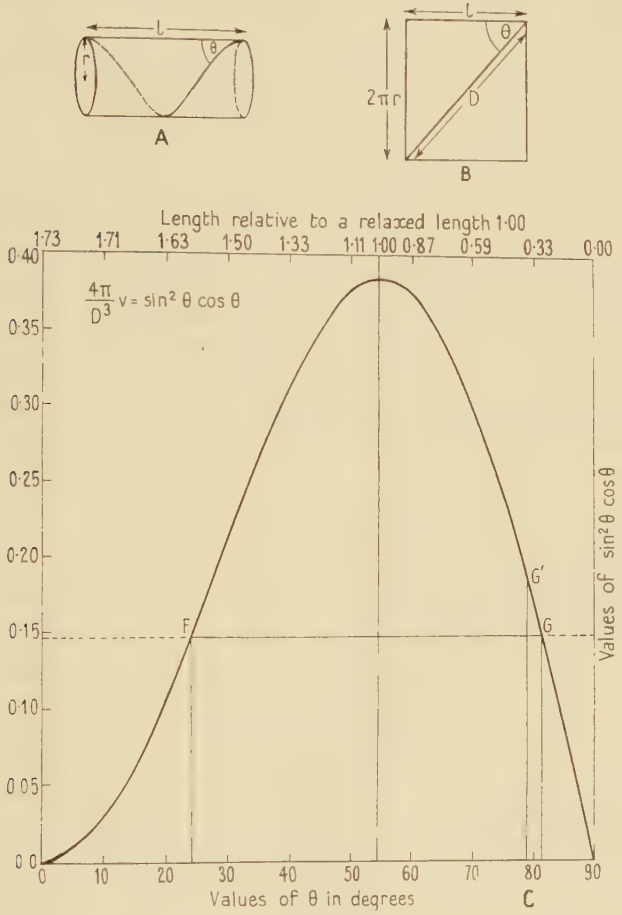

FIG. 9. A. Diagram of cylindrical element of unit length and circular cross-section with a single complete turn of the geodesic round it. B. The same element cut along its length and unrolled. C. Graph showing the relationship between the volume of the element and the value of the angle θ between 0° and 90°. The difference between G and G' in this figure has been deliberately exaggerated in the interests of clarity.

decrease in volume of the element. This condition almost obtains when the worm is full of ripe gonads.

In the relaxed position the volume of the cylindrical element in the living worm is not in fact maximal. It is elliptical in cross-section and not circular. Consequently it can both elongate and shorten, the limits being set by the points at which the cross-section must become circular in order to

accommodate the fixed volume of the element in the worm's body. These points are marked in fig. 9C as *F* and *G*.

We can calculate what these points must be. Suppose that at relaxed length the ratio of the semi-major to semi-minor axes, a/b, of the elliptical cross-section of the worm is n. The perimeter of the ellipse is:

$$2\pi\sqrt{\tfrac{1}{2}(a^2+b^2)} = 2\pi\sqrt{\tfrac{1}{2}a^2\left(\frac{n^2+1}{n^2}\right)}$$

whilst the volume of an element of length l_0 will be:

$$\pi abl_0 = \pi\frac{a^2}{n}l_0.$$

If this element of length l_0 is now inflated to its greatest possible volume, the perimeter remains constant but the cross-section becomes circular with radius r, such that:

$$2\pi r = 2\pi\sqrt{\tfrac{1}{2}a^2\left(\frac{n^2+1}{n^2}\right)}.$$

The volume now reached by the element of length l_0 will be

$$\pi r^2 l_0 = \pi\tfrac{1}{2}a^2\left(\frac{n^2+1}{n^2}\right)l_0.$$

It follows that the ratio of the actual volume of the element to its greatest possible inflated volume will be

$$\frac{\pi\dfrac{a^2}{n}l_0}{\pi\tfrac{1}{2}a^2\left(\dfrac{n^2+1}{n^2}\right)l_0} = \frac{2n}{n^2+1}.$$

It now follows that the limits of extreme extension and contraction will be set when the greatest volume of a cylindrical element at these lengths reaches this fraction:

$$\frac{2n}{n^2+1}$$

of the maximal value at relaxed length.

The value of n at relaxed (and minimum) length can be obtained from transverse sections of the animal. Stained and mounted sections are projected on to the viewing screen of a photomicrographic apparatus and direct measurements of $2a$ and $2b$ made. Observations on some ten relaxed worms without ripe gonads show that the average value for n at relaxed length is 5·1. The maximum and minimum values of n obtained at relaxed length were 5·6 and 4·7. Since $n = 5{\cdot}1$ we find:—

$$\frac{2n}{n^2+1} = 0{\cdot}39.$$

We can therefore draw *FG* in fig. 9C at a height corresponding to 0·39 of the maximum volume. In the arbitrary units of the ordinate of fig. 9C this corresponds to a value of 0·15.

By employing the relation $l = D \cos \theta$ we can construct a scale in fig. 9C relating the angle θ to the length of the worm relative to its resting length (taken as unity).

Given the demonstrated properties of the membrane system, if we determine by observation the ratio of the axes of the cross-section of the worm in the relaxed position, we are now able to predict the maximal and minimal possible length of the worm and the angle θ made by the geodesic fibre helices with the long axis.

The angle θ can be measured as follows. The specimens in their various conditions of contraction and extension are fixed and longitudinal sections cut in the place of the basement membrane. These sections are then impregnated with silver according to Wilder's technique, and mounted. The angle 2θ can be measured a number of times in each section by projecting it on to the screen of a photomicrographic apparatus. Thus an average value of θ for each particular worm used can be calculated. The following table gives the maximum and minimum values for θ found at maximum, minimum, and relaxed lengths.

	Minimum value	*Maximum value*
	θ	θ
Maximum length . . .	22°	24°
Minimum length . . .	70°	81°
Relaxed length . . .	52°	57°

Using the value, $n = 5{\cdot}1$, we may construct a table:

	Calculated values		*Observed values*	
	Length	θ	*Length*	θ
Relaxed length .	(1·0)	54° 44′	(1·0)	55°
Maximum length .	1·6	23° 30′	1·7	23°
Minimum length .	0·25	81° 45′	—	—
Length when $n = 1{\cdot}5$	0·27	81° 0′	0·3	75°

The agreement between the observed and calculated values for maximum length is evident. There is also agreement for the angle θ at relaxed length. If the membranes behaved as a frictionless, collapsible lattice of inextensible fibres the worm should in fact exhibit no relaxed length: it should remain at any length which it happened to occupy between the maximum and minimum. The fact that it assumes a resting length under anaesthesia and regains that length rapidly when released after passive stretching, shows that some small restoring force is being exerted. This restoring force is probably made up of two components, the tendency of the semi-fluid body constituents to spread

out as flat as possible and some slight elastic extensibility of the fibres and membranes. If such a restoring force, however small, acts symmetrically, the animal will tend to assume a shape in which θ becomes 55°, since any departure from this shape involves the performance of work. The agreement between the observed and calculated values of θ for worms in the relaxed position is thus in keeping with the observed properties of the worms.

The values at theoretical minimal length of the worm as limited by its volume cannot be reached since the worm is found to be unable to shorten to the point where its cross-section becomes circular. At real minimal length the cross-section is still elliptical. Observations on ten worms show that the major and minor axes stand in the ratio of about 3 to 2. That is $n_1 = 1{\cdot}5$.

Using this value of n_1 we can obtain from fig. 9C the corresponding value of θ, and the length relative to the relaxed length, in the following way. The element shown in fig. 9A will have shortened to a length l_1. Its volume will be

$$\pi \frac{a_1^2}{n_1} l_1,$$

where a_1 is the semi-major axis at this length l_1. If this unit is now inflated to its greatest volume at the length l_1, its volume will become:

$$\pi \tfrac{1}{2} a_1^2 \left(\frac{n_1^2+1}{n_1^2} \right) l_1.$$

The ratio of the volume of the inflated element to the volume of the natural element will be:

$$\frac{n_1^2+1}{2n_1}.$$

Since we know $n_1 = 1{\cdot}5$ this volume ratio $= 3{\cdot}25$ 3·0. All we need now do is to travel along the abscissa until we reach a point G^1 where the calculated volume on the curve is to the ordinal height of the line FG as

$$\frac{n_1^2+1}{2n_1} : 1.$$

This is found to occur when the fibre angle is 81° and the length of the unit relative to the relaxed length is 0·27. The distance between G^1 and G in fig. 9C has been exaggerated for clarity. The values calculated in this way are inserted in the table. It will be seen that they agree fairly well with the observed results.

The fact that the worm cannot shorten to the limit set by its volume is of interest. The actual limit is presumably set by the inability of the fibre lattice, whose units have a real thickness, to collapse indefinitely. As with a piece of garden trellis, collapse reaches a limit when the lattice components obliterate the spaces between them. Further shortening then is restricted to what can be obtained by buckling of the surface; a phenomenon seen in sea-anemones (Batham and Pantin, 1951), and in some terrestrial flatworms, where great contraction leads to the appearance of transverse ridges in the skin.

Acknowledgements

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REFERENCES

Batham, E. J., and Pantin, C. F. A., 1951. Quart. J. micr. Sci., **92**, 27.
Böhmig, L., 1930. *Kukenthal and Krumbach's Handbuch der Zoologie* — Nemertini.
Burger, O., 1895. Fauna und Flora des Golfes von Neapel, vol. xxii. Nemertinen.
——— 1897. *Bronn's Klassen und Ordnungen des Thier-Reichs.*
Wilder, H. C., 1935. Am. J. Path., **11**, 817.

The Tegumental Glands in the Land Isopoda

C. The Lobed Glands: The Properties of their Secretion and their Mode of Action

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Summary

1. The lobed glands of the Oniscoidea respond to appropriate stimulation by liberating droplets of secretion at the edges of the lateral plates and uropods.

2. The fresh secretion is a clear fluid, which is soluble in water, contains protein, water, perhaps fatty acid, but not mucin, fat, wax, or glycogen.

3. The uropod secretion is very sticky and viscous, slowly soluble in water, neutral in reaction, and without odour; the lateral plate secretion is a watery fluid, freely soluble in water, strongly acid in reaction, and with a pungent smell resembling that of butyric acid.

4. Both secretions solidify rapidly on exposure to air, and both are coagulated by alcohol.

5. Both types of gland secrete only in response to violent stimulation; this must be both strong and sudden in its application, and often causes fatal injury.

6. The uropod glands secrete more readily than the lateral plate glands.

7. The amount of secretion varies in different individuals of the same species; it is not correlated with the age, sex, or moulting condition of the animal, but may depend on its general condition.

8. The glands empty rapidly when stimulated, cannot repeat the process except at relatively long intervals, and appear to be incapable of secreting continuously.

9. They do not appear to be under nervous or hormonal control, but may be emptied passively by contraction of neighbouring muscles.

10. The significance of these results in relation to the problem of function is discussed.

Contents

Introduction

THE lobed glands of the Isopod Crustacea are remarkable structures which are peculiar to the group and very different in appearance from any glands found in other Crustacea. They are restricted absolutely to the

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Oniscoidea, i.e. to the terrestrial forms, but they appear to be universally distributed among the members of this sub-order, and are often present in great abundance. The Oniscoidea is one of the most advanced of the sub-orders of Isopoda, and since the lobed glands appear for the first time in the course of evolution in this group, and yet are here so well developed, it is difficult to believe that they do not perform some important function, probably one which is related in some way to the problem of living on dry land. Not the least remarkable feature of the lobed glands is that no one has ever succeeded in showing what that function is.

A critical review of previous work on the lobed glands, and a detailed survey of their structure and distribution in several genera, have already been given (Gorvett, 1951). Although this work was primarily concerned with the morphology of the glands, one of its objects was to discover whether the varying condition of the glands in the different species could be correlated with some other variable feature, either of the animal or of its environment, and so provide a clue to the problem of function. It appears, however, that no such correlation exists, and the main conclusions to be drawn from a study of comparative anatomy are that the lobed glands have evolved in adaptation to terrestrial life, but that their function is not connected in any way with the problems of respiration in air, adaptation to dry surroundings, moulting, or reproduction.

Other investigations have dealt more directly with the problem of function, and have been directed along two separate paths. First, an attempt has been made to determine more precisely the nature of the material secreted by the lobed glands, the conditions under which secretion takes place, and the mechanism of the process; for a knowledge of *what* the glands secrete and *how* they secrete it might well give some indication of *why* they secrete and what purpose they serve. The results of this part of the work are described in the present paper. Secondly, experiments have been designed to test more directly the validity of the various hypotheses relating to the functions of the glands. Descriptions of these, and a full discussion of the problem of function, will be given elsewhere.

Material and Methods

Most of the work was done on *Porcellio scaber* Latr. This is not only one of the most readily available species, and one of the most amenable to experimental work, but also, of the twelve species investigated (Gorvett, 1951), the one which shows the greatest development of the uropod and lateral plate glands.

Both groups of glands may be induced to secrete by almost any type of stimulation that is sufficiently violent; as shown below, it must be both strong and suddenly applied. Most of the material used in the following observations on the properties of the secretions was obtained either by firmly and rhythmically squeezing the animal dorso-ventrally with forceps, or by immersing it in alcohol. The material is available only in minute quantities (the droplet

on each lateral plate is rarely as big as the head of a one-inch pin), and this makes the investigation of certain of its properties extremely difficult.

Observations

Properties of the secretion

Previous workers have had very little to say about the properties of the material secreted by the lobed glands, and most have been content with the observation, first made by Lereboullet in 1853, that it is a viscous fluid which can be drawn out into fine threads like the silk of spiders. Herold (1913) added a few more details: he noted that the glands secrete when the animal is immersed in alcohol, and that the resulting secretion rapidly coagulates to an opaque white mass; he was also the first to point out that only the uropod secretion is viscous, the secretion from the lateral plates being a thin watery fluid.

Closer investigation has shown that, although the two secretions have much in common, this is by no means the only difference between them. The fresh secretions from the two types of gland (obtained by squeezing the animal) are both clear, colourless fluids, but they differ considerably from each other in at least three important properties. The first and most conspicuous of these is viscosity. The uropod secretion is extremely sticky and viscous, and can be drawn out into fine threads a foot or more in length. These threads rapidly 'set' in air, passing into a solidified condition, still flexible, but no longer extensible, differing in this last respect from the silk of spiders. The lateral plate secretion is a thin, watery fluid which cannot be drawn out into threads.

Secondly, the lateral plate secretion differs noticeably from the uropod secretion in having a peculiar, pungent odour, closely resembling that of butyric acid. Verhoeff (1907) also noted this acrid smell, and it is remarkable that Herold (1913) was unable to detect it. Verhoeff was mistaken, however, in supposing that the uropod glands are equally responsible, for the secretion from the latter appears to be completely odourless. The lateral plate secretion was tested for butyric acid by warming an alcoholic solution with sulphuric acid; the pineapple odour characteristic of ethyl butyrate seemed to be detectable, but this was not certain owing to the small amounts of material.

Thirdly, the secretions differ markedly in pH. Fresh lateral plate secretion has a strongly acid reaction, and treatment with a variety of indicators and test papers suggests that it has a pH of approximately 2·4, possibly less. Uropod secretion has a neutral or even faintly alkaline reaction, with a pH of about 7·4.

On exposure to air both secretions rapidly contract and harden. Within a few seconds of emergence from the ducts they change from clear liquids to translucent solids, the uropod secretion being somewhat gelatinous, the lateral plate secretion hard and wax-like. The process appears to be due to loss of water, since it is indefinitely delayed in a water-saturated atmosphere, and contact with cobalt chloride paper proves that water is undoubtedly

present in the fresh secretion. That it is not the result of an oxidation proces is further supported by the fact that it occurs equally rapidly in coal gas. Th hardened lateral plate secretion, even after five days' exposure to air, sti has a strongly acid reaction when dissolved in water, showing that the aci component is not readily volatilized. Immersion in alcohol has a simila solidifying effect, although the process probably involves an effect on th protein constituents as well as simple dehydration, and the resulting coagulur is somewhat different in its physical properties (solubility, for instance). A noted by Herold, the glands frequently secrete when the whole animal i immersed in alcohol, and the material from both types of gland then coagu lates, immediately after emerging from the duct openings, to a white flocculen solid.

Fresh lateral plate secretion is instantly and completely soluble in water fresh uropod secretion much less readily so. In both cases, secretion hardened by exposure to air is more slowly soluble, and secretion solidifie in alcohol appears to be completely insoluble in water. Both secretions ar completely insoluble in alcohol and fat solvents (ether, chloroform, acetone xylol). In cold concentrated hydrochloric acid, secretion solidified in alcoho becomes transparent, gelatinizes, and apparently dissolves after several hours it also seems to dissolve in hot potassium hydroxide solution, but the result of both of these tests are somewhat uncertain.

Protein colour tests may be applied fairly readily to droplets of secretion smeared on glass microscope slides. Both secretions give positive reaction with the xanthoproteic, Millon's, arginine, and biuret tests (the biuret tes is uncertain in the case of the lateral plate secretion), doubtful reaction with the glyoxylic acid test, and negative reactions with the aldehyde, sulphur and Molisch tests. It may be concluded that both secretions contain protein with phenol group and arginine, but no tryptophane, sulphide, or carbo hydrate. The presence of protein is confirmed by the fact that alcohol solidified secretion is soluble in pepsin and trypsin solutions. The waxy appearance of the hardened secretion suggests that wax may be present, but treatment of alcohol-hardened secretion with ether in a Soxhlet apparatus for three hours produces no change in its appearance.

Other colour-tests may be applied to material which is smeared on micro scope slides and then coagulated with alcohol to prevent its being dissolved in water. Both secretions readily take up Delafield's haematoxylin, but while the uropod secretion is deeply stained with acid dyes such as eosin and acid fuchsin, lateral plate secretion is unaffected. Both are stained yellow with osmium tetroxide, and faintly blue with nile blue, but not at all with sudan III. Thionin, mucicarmine, mucihaematin, and Best's carmine all give negative results. Thus the secretions may contain small amounts of fatty acid, but no triglyceride, mucin, or glycogen. Ide, Herold, and others have observed differences in the degree of staining of different glands in sectioned material, and Herold suggested that these were due to the varying distances of the glands from the surface and consequent differences in the effects of the

xative; it is clear that they are at least partly due to differences in the roperties of the secretions from the two types of gland.

The two secretions show a striking difference in their reaction to alkalis. Uropod secretion is not visibly affected, but in contact with the hydroxides f sodium, ammonium, calcium, &c., lateral plate secretion acquires a vivid emon yellow colour. The reaction is reversible and appears to depend solely n pH. It occurs with alcohol-hardened secretion and with secretion hardened y exposure to air and left for several days, and is therefore due to a non-olatile constituent of the secretion. It may also occur on the body of the nimal: if an animal is squeezed to induce liberation of secretion and then laced in a moist atmosphere to prevent solidification, the droplets gradually urn yellow, presumably as a result of the gradual diffusion of the secretion nto the calcified layer of the integument. The reaction never occurs with ecretion alone on a slide, or in the case of an animal exposed to dry air, nd never with uropod secretion. The lateral plate glands themselves turn ellow during fixation in alcohol, or during anaesthetization with chloroform, robably owing to an increase in the permeability of the gland walls, and ither an outward diffusion of secretion into the integument, or a mingling f secretion with alkaline constituents of the blood (which has a pH of ver 8·0).

This yellow coloration (which is not shown by butyric acid) suggests the resence of nitrophenols—acids which combine with alkalis to form intensely ellow salts—but these appear to be entirely unknown in living organisms. The occurrence of flavones would be less surprising, for although these are lant pigments, they are yellow in colour and water-soluble, and they are nown to occur in insects; they are taken in with the food, and either deposited n various parts of the body or, in some cases, secreted by the spinning lands (work summarized by Wigglesworth, 1950). Their colour, however, s intensified with *acid*, not with alkali. The reaction is not, perhaps, a very elpful clue to the composition of the secretion, for it appears to be charac-eristic of several compounds with conjugated double bonds.

An attempt was made to determine the iso-electric point of the uropod ecretion, since from some of its reactions this substance appears to be of an mphoteric nature. Samples of secretion were soaked in buffered solutions f various pH values to which were added either potassium ferrocyanide or opper sulphate, then washed and treated with ferric chloride and potassium errocyanide respectively. The colour reactions were very irregular and gave o indication of an iso-electric point.

When fresh secretion is heated in a flame, it contracts and hardens, turning rom a clear fluid to an opaque white solid, then to black, finally burning ompletely away.

The more important properties of the secretions of the lobed glands, herefore, appear to be as follows. The fresh secretion from both types of land is a clear fluid, soluble in water, containing protein, water, and possibly ome fatty acid. It contains no trace of mucin, fat, wax, or glycogen. On

exposure, even in the absence of oxygen, it contracts within a few second to form a hard translucent solid, apparently by loss of water. It is coagulate by alcohol to a white solid, insoluble in water, alcohol, and most othe solvents. The chief differences between the secretions from the latera plates and from the uropods are that while the uropod secretion is extremel sticky and viscous, only slowly soluble in water, neutral or faintly alkaline i reaction, and without detectable odour, the lateral plate secretion is a thi watery fluid, freely soluble in water, strongly acid in reaction, and with pungent smell resembling that of butyric acid.

Conditions under which secretion occurs

In the hope of throwing some light on the natural functioning of the lobe glands, an investigation has been made into the various conditions whic bring about a liberation of secretion. Secretion has never been observed, an there is no evidence that it ever takes place, except when the animal has bee stimulated by some change in its external environment. Previous author have noted that both mechanical and chemical stimulation (immersion i fixing fluid, for instance) may induce secretion, and the experiments describe below suggest that the glands react in a similar way to violent changes i most environmental conditions.

The amount of material secreted varies considerably from one individua to another, but in general the maximum amount for each species appears t correspond with the relative development of the glands; it is most copious for instance, in *Porcellio scaber*, less abundant in *Oniscus asellus*, still less i *Armadillidium vulgare*, while *Ligia oceanica* never produces a secretio under any conditions. In experiments with *Porcellio scaber* it was found tha the uropod glands secrete more readily than the lateral plate glands. Abundan secretion from the uropods is often accompanied by no secretion, or onl slight secretion, from the lateral plates, or from some lateral plates only, an very rarely do the lateral plates secrete alone. The rate and amount of secretio seem to vary with the individual rather than with the type or strength c stimulus, or with such clearly defined features as the age, sex, and moultin condition of the animal. Animals which have received the same treatmen for many weeks, and which resemble each other in the features mentione above, often differ considerably in the amount of secretion they produce This is true even when all the individuals receive an identical stimulus, a must presumably be the case when, for instance, they are immersed i alcohol. It is possible that the amount of secretion may depend on the genera condition or vigour of the animal, for it is noticeable that lively individuals which struggle vigorously after immersion in fixing fluid, almost invariabl produce more secretion than the more feeble ones.

The conditions investigated include chemical stimulation, pressure, shaking heat, cold, and electric shock. When a woodlouse is plunged into fixing fluid the glands usually secrete a few minutes afterwards, although they do no invariably do so. The slight delay in the reaction of the glands presumabl

represents the time taken for the fixing fluid to penetrate the integument. Secretion occurs in most fixing fluids, including ethyl alcohol of any strength from 70 per cent. upwards. With Bouin's fluid secretion seems to be less abundant and less rapidly produced. After secreting in alcohol, the animal can sometimes be revived by washing in water and replacing in air.

For histological work it is necessary to prevent this emptying of the glands during fixation. Preliminary anaesthetization with chloroform or ether is useless for this purpose, for not only is immersion in liquid chloroform often followed by abundant secretion, but some individuals secrete when subjected to chloroform vapour (especially when dilute), and others may secrete after subsequent immersion in alcohol. Fortunately, secretion may be effectively prevented by fixing very rapidly in hot alcohol.

Secretion may very often be induced by grasping the abdomen dorso-ventrally with forceps and squeezing firmly and rhythmically. Indeed, this is the simplest and surest method of obtaining secretion for test purposes, although (as with chemical stimulation) it is by no means invariably effective, and animals which do not secrete in response to squeezing often do so when subsequently placed in alcohol. Secretion from the uropod glands is usually the first to emerge, while droplets may appear on some or all of the lateral plates at the same time, or after a short interval, or not at all, even after complete demolition of the abdomen. The reaction to stimulation varies enormously in different individuals, but, as already mentioned, does not appear to be correlated with any external condition. In some cases the pressure required to induce secretion is such as to cause fatal damage to the animal. In others the glands respond to slighter pressure, and the animal survives the experiment and may be made to repeat the process, but even with animals of this type fatal injury can be avoided only by very careful manipulation. In more carefully controlled experiments animals were placed between two glass slides and subjected to gradually increasing pressure, either dorso-ventrally or laterally, and in each case it was found that uropod secretion did not appear until the integument was actually fractured, and that lateral plate secretion was slight and less frequent. Thus, to induce secretion, the increase in pressure must not only be sufficiently great, but also sufficiently rapid, as during the rhythmical squeezing of the abdomen or at the moment of sudden fracture with the glass slides.

Collinge (1921) mentions that secretion is more copious when the animals are disturbed, and the reaction of the glands to chemical and mechanical stimulation suggests that they may respond to any general disturbance of the body. A simple mechanical disturbance, such as shaking, has no effect, however. Two animals were placed in corked four-inch glass tubes attached to a mixing machine. At first they were found clinging to the corks, so these were replaced by caps of tinfoil. The tubes were revolved at the rate of forty revolutions per minute for five minutes, i.e. the animals were thrown from end to end of the tubes four hundred times in five minutes. No trace of secretion appeared either on the uropods or on the lateral plates, and there is no

evidence that such disturbance causes the glands to respond any more readily than usual to subsequent stimulation of a different type. Evidently shaking does not provide a stimulus sufficiently strong to activate the glands.

It was noticed incidentally in the course of other work that the application of heat often causes active secretion from the lobed glands. In certain experiments, for example, it was necessary to support the animal in mid-air by attaching it to the head of a pin with melted wax, and in many cases secretion occurred at the moment when contact was made between the body-wall and the heated wax, especially when this was applied to the ventral surface. One animal reacted dramatically when a hot scalpel accidentally touched one of its antennae by the immediate and simultaneous secretion of large droplets on all the lateral plates, although the uropod secretion on this occasion was unusually slight. Similar, though less spectacular, results were obtained with other animals.

If warmed in a test-tube, the animal runs about vigorously at first, but soon turns on its back in a state of coma and dies without liberating any visible secretion. Animals placed in an oven at 60° C., in either damp or dry tubes, die after some hours without secreting. In every case it appears that secretion takes place only when the heat is applied suddenly, and that gradual increase of temperature, even to a higher level, is ineffective.

Low temperatures, unlike high ones, do not appear to induce secretion, probably because the change from room temperature cannot be made sufficiently great or sufficiently rapid in comparison with high temperatures to provide the necessary stimulation. Certainly, animals placed in a refrigerator and kept there for several hours showed no signs of secreting, although they could be induced to do so later by squeezing the abdomen.

As further evidence of the influence of violent stimulation on the lobed glands, the effect of electric shock may be mentioned. An animal was fixed ventral side uppermost by attaching the head of a pin to the dorsal side with wax and inserting the pin in a cork. The shallow depression formed by the head, lateral plates, and telson, was filled with water, and the tips of a pair of electrodes from an induction coil were then placed in this. At the moment of closing the circuit there was an instantaneous and abundant secretion from the uropods, but none from the lateral plates, although the animal was killed by the shock. The experiment was repeated with the same result.

Thus it appears that the lobed glands are induced to pour out secretion by almost any violent stimulus, but that they do not react to any stimulus, however strong, unless it is also sudden in application—i.e. unless it reaches its maximum strength almost instantaneously. It has already been pointed out that the conditions required to bring about secretion are so violent that, unless very carefully controlled, they usually cause the animal to die. It seems extremely probable that if the functioning of the glands under natural conditions depends on such stimulation, their use must be followed almost invariably by the death of the animal. The glands never react to minor injury such as pinching or removal of antennae or legs.

Mechanism of secretion

An attempt has been made to obtain information about the mechanism of the secretory process in the lobed glands, for although a knowledge of their mode of action may not provide conclusive evidence for a particular function, it may at least exclude the possibility of others.

It has been shown experimentally that the lobed glands produce visible secretion only when the animal is subjected to certain external conditions, i.e. they secrete only in response to stimulation. When suitably stimulated, the glands pour out material rapidly, completing the process in a few seconds, and they cannot be made to repeat it, even partially, in less than several hours. They need several days to recover completely, and if made to secrete three or four times in succession at twenty-four-hour intervals, the secretion (particularly in the case of the uropod glands) is noticeably more scanty, more watery, and less viscous than usual. They also appear very shrunken in section when fixed immediately after secreting. These facts suggest (1) that the gland is normally filled with fully formed secretion; (2) that this secretion is produced very slowly; (3) that, with appropriate stimulation, the secretion can be liberated almost instantaneously; (4) that the effect of stimulation is to cause the gland simply to liberate material already elaborated and stored, not to elaborate new material. It may be concluded that, whatever may be the function of the glands, liberation of fluid secretion can never be a continuous process and cannot be repeated except at somewhat infrequent intervals.

The lobed glands thus differ in certain respects from such glands as the mammalian salivary glands, in which the process of secretion is much more prolonged, and can be repeated at short intervals. Parasympathetic stimulation of these, moreover, appears to promote the taking up of water and salts from the blood, and at least the final stages in the elaboration of some of the more complex constituents from their precursors, as well as the liberation of the final product.

Little can be said about the precise mode of storage of the secretory material. There is no special reservoir in the gland, and the appearance of the duct and its branches does not differ noticeably in sections of full and empty glands. This is not true of the cytoplasm, however, which is considerably shrunken in sections of glands fixed after secreting, and it would seem that it is here that the secretion is stored. Ide (1891) described and figured large vacuoles filled with secretion, but these have rarely been seen, and then only after poor fixation; if vacuoles are present in the living gland, they are probably very small, and they do not appear in sections of fixed material. The stored material could be liberated simply by changes in permeability of the walls of the duct or of the vacuoles, or by an increase in pressure on the secretory fluid, either by contraction of the gland cytoplasm (there are no muscle fibres) or by external pressure.

There is also little positive information about the mode of control of the

secretory process. It has been shown that stimulation of the whole anim in a variety of ways causes the lobed glands to secrete, but it is not easy t determine exactly how the effect of the external stimulus is conveyed to th glands. There appear to be three possible mechanisms—nervous, chemica and mechanical.

The almost instantaneous response to many types of stimulation, and th frequently simultaneous action of all the reacting glands, suggest that a nerv reflex may be involved, but most of the evidence is against this view. Respons to some types of stimulus, particularly chemical, is often delayed, and th glands often react, not simultaneously, but in successive batches, or quit irregularly. Furthermore, it has already been pointed out that after narcotiza tion in chloroform many animals secrete when subsequently placed i alcohol, showing that secretion is not inhibited by inactivation of the nervou system. It is similarly unaffected by cutting or removal of the ventral nerv cord (unless, as sometimes happens, secretion takes place as a reaction to th cutting operation itself). Immersion in alcohol may still induce secretion, a least from the uropods, after section of the cord by a cut through the ventra body-wall, when the whole abdomen is cut away from the rest of the body and even when one side of the body has been removed, together with it attached uropod, but without any part of the ventral nerve cord. Tests in volving immersion in alcohol may not be conclusive, for it seems likely, a suggested below, that chemical stimulation (unlike other kinds of externa stimulation) may act directly on the glands without the intervention of a internal control mechanism. When the above experiments were repeated, however, and the animal was stimulated either by squeezing the abdomen or by thrusting a needle through the viscera, it was found that the glands secreted as before even if the animal appeared to be fully narcotized and in capable of showing any visible muscular response to stimulation.

In any case, the view that secretion may be under nervous control is strongly opposed by the histological evidence. When whole animals are treated (by immersion or injection) with gold chloride or methylene blue, then either dissected or sectioned, or when dissociated glands are similarly treated, no trace of a nerve supply to the glands can be detected, although nerves running to other parts of the body are clearly visible. It seems highly improbable, then, that the secretory process is under direct nervous control, although it may, perhaps, be linked in some way with other internal conditions such as muscle contraction or blood-pressure, which are themselves controlled by the nervous system.

Since a single localized stimulus may induce secretion from glands in widely separated parts of the body, sometimes from all the groups of lobed glands simultaneously, it is possible that they may be reacting to some chemical substance, perhaps to a specific hormone, carried in the blood-stream. The lobed glands undoubtedly expose a large surface-area to contact with the surrounding blood, although, as has already been pointed out, such an arrangement may be necessary for other reasons. However, the response of

the various glands to stimulation is often not simultaneous, and they may respond independently and at irregular intervals. Furthermore, when the response is simultaneous (as with heat stimulation), it is often almost instantaneous, and it is difficult to believe that such a rapid reaction could be brought about by chemical means in an animal with the relatively inefficient circulatory system of a crustacean.

The experimental evidence also fails to support the suggestion of a chemical mechanism. A drop of blood extracted by means of a fine glass capillary tube from the heart of a secreting animal does not induce secretion when injected into a second individual. Negative results in experiments of this kind may not be very significant, but more positive evidence may be obtained in other ways. For instance, squeezing the abdomen still induces the usual secretion in animals that have been visibly distended by injecting water or salt solution into the haemocoele, although it seems probable that the blood will have been considerably diluted, and the concentration of any stimulating chemical constituent reduced to well below threshold value.

Secretion after immersion in fixing fluids may well be an example of response to chemical action, but this appears to be a case of direct response by the glands to the external stimulus, and therefore not comparable with humoral control. Secretion does not begin until several minutes after immersion, usually only after all visible movement has ceased, and the glands tend not to act simultaneously; the lateral plate glands almost always begin to secrete only when the uropod glands have finished, and the reactions of the various groups are usually very irregular. It would appear that the delay is due to the time taken by the fixing fluid to penetrate the body-wall, and that the glands are here reacting to direct contact with the fixing fluid, which may either increase permeability or cause contraction of the cytoplasm. It is probable that the fluid will penetrate the thin-walled gill region more rapidly than any other part of the body, and so will affect the uropod glands, and cause them to secrete, before reaching the lateral plate glands. The same explanation would also account for the slower response of other genera, such as *Armadillidium*, which have thicker and less permeable integuments.

The effect of squeezing the abdomen suggests a third possibility—that, when liberating secretion, the gland may not be performing an active process at all, but passively submitting to external pressure. The expulsion of secretory material and the shrinkage of the gland during the process may, perhaps, be due to increased pressure of the surrounding blood, or contraction of muscles in the surrounding tissues (which may, in turn, increase the local blood-pressure). Since squeezing is most effective when applied to the abdomen, and particularly in the region of the heart, it may act directly on the uropod glands, and indirectly (by increasing the blood-pressure) on the lateral plate glands. However, this does not appear to be the case, for there is usually a slight time-lag between the application of pressure and the appearance of secretion, even in the case of the uropod glands, and the process continues for some seconds after the pressure is released. Also, by injecting salt solution into the

haemocoele, it is possible to raise the internal pressure to such an extent that the body is considerably distended and the inter-segmental membranes fully stretched, without inducing secretion, and the lateral plates can be pinched until completely crushed without producing any response in the glands they contain. There can certainly be no question of external pressure acting on the glands when these respond to such stimuli as heat and electric shock.

The alternative suggestion mentioned above, that secretory material may be extruded from the glands by the contraction of neighbouring muscles, seems, at first, rather improbable, but is not incompatible with the facts. All the glands appear to be closely adjacent to powerful dorso-ventral muscles; the amount of secretion produced seems to be correlated with the degree of struggling, i.e. muscular activity, which the animal undergoes during fixation; secretion is often accompanied by convulsive contractions of the appendages; the various forms of violent stimulation which result in secretion are all such as might be expected to induce convulsive contractions of the general musculature of the body, either with or without the intervention of the nervous system. The lobed glands are a comparatively recent development in the history of the Isopoda, and have presumably evolved from ordinary epidermal cells which have no nerve connexions. It would not be surprising to find that the glands were still dependent for their functioning on a clumsy device of this sort, and that a really efficient method of control had yet to evolve.

It has thus not been possible to obtain much positive information about the mechanism of secretion, but the evidence available seems to justify the following conclusions. (1) The secretion is produced very slowly, and, after emptying, the gland takes several days to refill with normal secretion. (2) The gland stores secretory material in its cytoplasm, and reacts to the appropriate stimulus by pouring out the whole of this stored secretion within a few seconds. (3) Secretion can never be a continuous process, and can be repeated only at relatively long intervals. (4) Liberation of secretion is almost certainly not under nervous control, is apparently not induced by a hormone or other chemical substance in the blood, or by increase in pressure of body fluids; the glands may be emptied passively by the pressure of contracting muscles in the neighbouring parts of the body, and are probably affected directly by fixing fluids.

Discussion

The anatomy of the lobed glands, their mode of action, and the properties of their secretion have now been described, but the question of function remains unanswered. As already shown, comparative anatomy provides few clues to the problem, and while indicating that the glands have evolved in adaptation to terrestrial life, discourages any suggestion that they may be concerned with respiration, prevention of desiccation, reproduction, or moulting.

The physiological information given in the present account is almost equally unhelpful, and arguments based on it are as inconclusive as those

based on anatomy. For example, the secretions from both types of gland have properties which are presumably distasteful to other animals; they are liberated rapidly, and apparently only in response to external stimulation; but the severity of the stimulus needed, the consequent ill effects of this on the animal, and the apparent lack of an efficient controlling mechanism make it impossible to accept the defence theory without strong additional evidence. The secretions harden rapidly on exposure, and appear to be incapable of spreading over the body surface; although soluble in water, they have no particular affinity for it, and allow it to evaporate rapidly in dry air; the glands are apparently incapable of secreting continuously, or of secreting at all without external stimulation: all these facts add to the anatomical evidence opposed to the respiration and anti-desiccation theories, but still do not provide conclusive proof. It seems unlikely that glands concerned in any way with ecdysis could be made to secrete in response to external stimulation, or that they would pour out their secretions on the surface of the body, or that they would function equally well at all stages of the moulting cycle; but, again, the evidence is largely negative. Similar arguments could be advanced for or against other theories, but they would be equally inconclusive.

Thus the physiological evidence, like the anatomical, is largely circumstantial, and there is, so far, no final and positive proof of the soundness, or the falsity, of any particular view. This is largely true, also, of the evidence from experiments designed to test the various theories more directly. In order to find a satisfactory solution to the problem of function, it will therefore be necessary to describe these experiments, and then to review the various possibilities and weigh very carefully all the evidence for and against each one. An account of the experimental work, and a full discussion of all the available evidence, will be given in a separate paper.

REFERENCES

Collinge, W. E., 1921. Ann. Mag. nat. Hist., **7**, 212.
Gorvett, H., 1951. Quart. J. micr. Sci., **92**, 275.
Herold, W., 1913. Zool. Jahrb. Jena (Anat.), **35**, 457.
Ide, M., 1891. La Cellule, **7**, 345.
Lereboullet, A., 1853. Mém. Soc. nat. Hist. Strassb., **4**, 1.
Verhoeff, K. W., 1907. S. B. Ges. naturf. Fr. Berl., 229.
—— 1908. Arch. Biont. Berlin, **2**, 335.
Wigglesworth, V. B., 1950. *The principles of insect physiology*. London (Methuen).

Studies of the Histology of the Mid-gut of the Chelonethi or Pseudoscorpiones

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Summary

1. The histology of the mid-gut of *Dactylochelifer latreilli* (Leach) is described. The mid-gut is divided into two regions: an anterior diverticular region consisting of digestive cells, excretory cells, and small basal cells of unknown function, and a narrow, posterior post-diverticular region possessing a syncytial epithelium. The whole mid-gut is invested in a layer of peritoneal cells.

2. The changes in the staining reaction of protein globules undergoing intracellular digestion are followed.

3. The development of excretory cells is described. The function of the post-diverticular mid-gut in the excretory process is outlined.

4. Storage of fatty material and glycogen occurs in the digestive cells and in the peritoneal epithelium.

5. The structure and functions of the false scorpion mid-gut are compared with those of other Arachnids.

Introduction

THE general morphology of the gut of false scorpions has been described adequately by Croneberg (1888) for *Chernes hahni* and by Kästner (1927) for *Neobisium muscorum*. The narrow fore-gut expands behind the brain into a mid-gut consisting of two regions, which are morphologically and physiologically different. The anterior or diverticular region consists of a broad central tube from which lead six pairs of lateral diverticula and one backwardly directed ventral diverticulum. The last pair of lateral diverticula is subdivided into four pairs giving nine pairs of diverticula on each side. As in other Arachnida the diverticular mid-gut fills much of the abdomen and part of the cephalothorax. Unlike those of most Arachnida, however, the diverticula are broad pouches. The second region, the post-diverticular mid-gut, is a narrow tube leading backwards from the central tube of the diverticular region in the form of an ascending S. The upper or final limb of this expands distally into a stercoral pocket. No Malpighian tubules are present in false scorpions.

The histology of the diverticular mid-gut has been variously described. Croneberg (1888) and Bernard (1893) maintain that it is composed of one type of cell while Bertkau (1887) and Bruntz (1904) state that there are two. Kästner (1927) endorses the latter opinion; he found large cells which he

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believed were concerned with intracellular digestion, and smaller less numerous cells filled with spherical inclusions. The role of the latter cells he does not specify but he mentions that similar cells in spiders secrete enzymes into the gut lumen. The post-diverticular mid-gut is generally stated (Kästner, 1927; Roewer, 1937) to have a uniform cellular epithelium throughout. Kästner suggests that a possible function is the absorption of nutrient material which has passed from the digestive region of the gut. The peritoneal epithelium is one cell thick over most of the diverticular region; between the posterior diverticula and the post-diverticular intestine it is several cells thick and has a packing function. Both Croneberg (1888) and Bernard (1893) point out that this epithelium is a site of food storage, and call it the 'fat body' although it is not homologous with the true fat body of insects.

Material and Methods

Two species of false scorpion were used in these studies: *Dactylochelifer latreilli* (Leach), collected on sand-dunes along the Suffolk coast, and *Neobisium muscorum* (Leach), collected in the Manchester district. Both were collected by hand-sorting suitable material. In the laboratory the animals were kept in cork and glass cells as described by McIntire (1868), and fed on *Drosophila* larvae.

The most reliable fixative for the general histology of the gut was found to be Heidenhain's Susa. Bouin's fluid was also satisfactory but the results obtained with Carnoy's fluid and Carnoy-Lebrun were poor. Saline Flemming—without-acetic and Champy's fluid fixed the gut tissues well but the region of optimal fixation was small. Before fixation some sclerites were removed from the abdomen to allow more rapid penetration of the gut. Most material was treated after fixation by Peterfi's double embedding method (Pantin, 1948). The material was subsequently embedded in paraffin wax, melting-point 54° C. Most of the material was sectioned at 8 μ.

For investigating the changes of the gut epithelium after a meal, individual *D. latreilli* were placed separately in small cages, fed on one *Drosophila* larva and then starved for 12 days. They were then fed again with a *Drosophila* larva and fixed at an appropriate interval after the beginning of feeding. The specimens were not kept in a constant temperature chamber but the temperature of the drawer in which they were kept was fairly constant at 15–16° C. At least two individuals of *D. latreilli* were fixed in Susa at each of the following times after the beginning of feeding: $\frac{1}{2}$ hour, 2 hours, 4 hours, 14 hours, 24 hours, 40 hours, 4 days, 5 days, 7 days, 9 days, 12 days, and 1 month. Feeding was regarded as beginning when the larva was drawn on to the mouthparts by the two chelicerae. An adult *D. latreilli* usually rejects a final instar *Drosophila* larva after about 50 minutes' feeding.

Two staining methods were used on the Susa-fixed material. Some sections were taken from the sequence on the ribbon and stained with Heidenhain's iron haematoxylin and differentiated in a saturated solution of picric acid. Alcoholic eosin was sometimes used as a counterstain. The main series of

sections was stained by the quadruple method outlined by Millot (1926). Millot does not give full details of the method but the following scheme was found to give good results with Susa-fixed material:

Reagents

Haemalum (G. T. Gurr's)
Acid fuchsin, 2 per cent. solution in 40 per cent. alcohol
Metanil yellow, 1 per cent. aqueous solution
Light green, 0·125 per cent. aqueous solution

Method

1. Remove wax; bring slide to water.
2. Haemalum, 15 minutes at room temperature.
3. Wash in water, 2–3 minutes.
4. Acid fuchsin at 30° C., 20 minutes.
5. Forty per cent. alcohol at room temperature, 1 minute.
6. Rinse in water, 10 seconds.
7. Metanil yellow at room temperature, 5 minutes.
8. Rinse in water, 10 seconds.
9. Light green at room temperature, 45 seconds.
10. Rinse in water, 5 seconds.
11. Dehydrate through 50 per cent., 90 per cent., and absolute alcohols to xylene in 45 seconds.
12. Mount in balsam.

To obtain comparable results, these times were rigidly adhered to, except at the haemalum stage. The method stains nuclei blue, ground cytoplasm green, and cellular inclusions green, yellow, red, or intermediary colours. There is a tendency for the metanil yellow to stain the ground cytoplasm. A disadvantage of the method is a tendency for the sections to fade.

After trying Millon's reagent and the biuret and xanthoproteic reactions on frozen sections it was found that the most satisfactory 'protein' test was the modification of Sakaguchi's method for demonstrating arginine and related compounds, described by Baker (1947). One slight modification of Baker's method was made: the colour of the sections was stabilized with glycerine and the sections mounted in this medium. This technique was used by Serra (1946) in his modification of the Sakaguchi reaction. The test was carried out on sections taken from the main Susa-fixed series, cut at 8 μ.

In order to fix glycogen *in situ* the picric acid-alcohol-formalin mixture described by Rossmann (1940) was used. This was found to be better than Bouin-Allen (Bolles Lee, 1937) and the Pasteels-Leonard fixative (Lison, 1936). Fixed material was treated by Peterfi's double embedding method (Pantin, 1948) and embedded in paraffin wax, melting-point 54° C., for 2½ hours at 56–57° C. The embedded material was cut at 8 μ. Parts of the ribbon were used for a saliva control, parts for general staining, while the remainder

was stained to show glycogen. Bauer's method (Lison, 1936) was used to demonstrate glycogen in the sections. The iodine method as used by Gage (Bensley, 1939) was employed on some sections; this method although useful for confirmation has the disadvantage that the petroleum jelly mountant makes the sections unsuitable for high-power observation. Sections from both *Neobisium muscorum* and *Dactylochelifer latreilli* were stained to show the distribution of glycogen.

Material intended for the investigation of fat storage was fixed in 6 per cent. commercial formaldehyde (neutralized with magnesium carbonate) in 0·75 per cent. saline solution. It was subsequently embedded in gelatine and the blocks stored until required in 2 per cent. formaldehyde solution over magnesium carbonate. Sections were cut at 20 μ on a freezing microtome and treated with sudan black (Lison, 1936).

The Histology of the Mid-gut

The diverticular mid-gut has an epithelium composed of three types of cell. The largest of these, which is concerned with the intracellular digestion of the food, will be called the digestive cell. Another cell type, also large, has an excretory role and will be called the excretory cell. The ratio of the number of digestive cells to the number of excretory cells is approximately 7:4. As both excretory and digestive cells vary considerably in appearance at different stages, only one stage of each will be described in this section. The third type of cell occurs between the bases of the other two. It is much smaller than the digestive and excretory cells, and its function is unknown. All three types of cell occur both in the central tube and in the diverticula.

After 12 days' starvation the digestive cell is devoid of protein inclusions but usually contains two nuclei, one basal and one apical (fig. 1). The cytoplasm is sparse with large empty spaces probably occupied by fatty material in life. Sudan black treatment of frozen sections shows large quantities of fatty material in the digestive cells, and fixation by osmium tetroxide reveals considerable amounts of osmiophile material. At this stage the height of the digestive cells in a mature *D. latreilli* is about 100 μ, although in some instances they may be as much as 135 μ high. The width of the cells is greatest subapically, being here about 18 μ. As pointed out by previous authors the cells are club-shaped. The cells are attached at the base only, there being no lateral protoplasmic communication in the epithelium.

The excretory cells are smaller than the digestive; they are between 50 μ and 100 μ high and elliptical, in their most frequently observed state (fig. 1). At this stage the cell is filled with spherical inclusions staining intensely with acid fuchsin. These inclusions, which vary in size from 2 to 12 μ, seem to occur in a large apical vacuole. The single nucleus of the cell is usually situated basally and is somewhat larger than those found in the digestive cell. The cytoplasm of the excretory cells, which is concentrated basally, stains more deeply than that found in the digestive cells. Like it, however, it stains with light green by the quadruple method given.

The third type of cell occurring in the diverticular mid-gut of *D. latreilli* is much smaller than the other two and is found between their bases against the peritoneal layer bounding the gut (fig. 1, basal cell). These cells are about 12 μ long with a conspicuous nucleus containing numerous scattered chromatin granules. Sometimes the cells are found singly but more often they occur in groups of three or four. The function of these cells is unknown; it is possible that they are regenerative cells although nothing has been observed to support this idea.

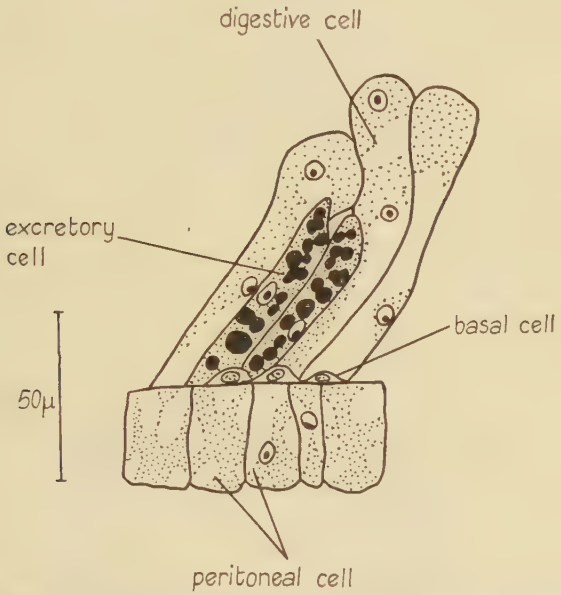

FIG. 1. *D. latreilli*. Wall of diverticular mid-gut after 12 days' starvation. Susa: haemalum/acid fuchsin/metanil yellow/light green.

The post-diverticular intestine and the stercoral pocket consist of a syncytial epithelium throughout. The tube can be divided into three regions on the basis of the structure of the epithelium. The following description refers to the resting state when nothing is passing along the tube. The first region extends along the ventral limb of the tube as far as its junction with the middle limb. The epithelium of this region is about 15 μ high and bounds a round or oval lumen (fig. 2). The cytoplasm is dense, sometimes appearing striated. Nuclei are numerous; some pairs of smaller, contiguous nuclei with scattered chromatin granules suggest that nuclear division occurs in the epithelium.

The next region extends along the middle limb and part of the way along the dorsal limb. Unlike the first region the epithelium is very uneven, being pitted and furrowed so that in some sections it appears papillate. From some of the epithelial lobes protoplasmic strands stretch across the lumen to join the epithelium on the other side; in some sections there appears to be a protoplasmic web across the lumen. The cytoplasm of the epithelium stains deeply

at the base, but towards the lumen it thins, making it difficult in some instances to define the limit of the epithelium (fig. 3).

Towards the posterior end of the dorsal limb the epithelium becomes truly papillate and loses the protoplasmic filaments. This hind region comprises

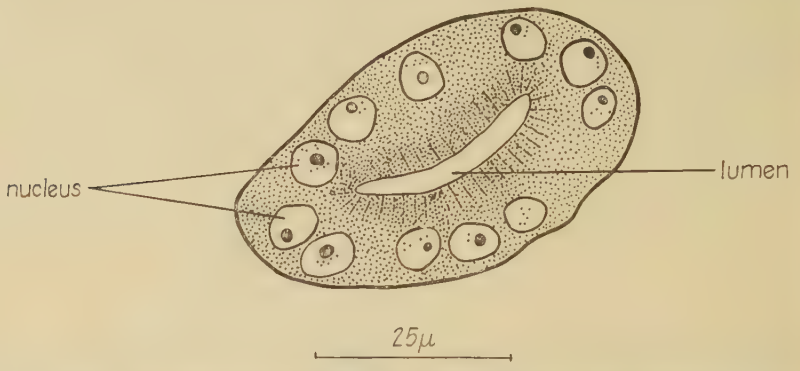

Fig. 2. *D. latreilli*. Post-diverticular mid-gut, first region, inactive phase, showing cytoplasmic striation. Susa: haemalum/acid fuchsin/metanil yellow/light green.

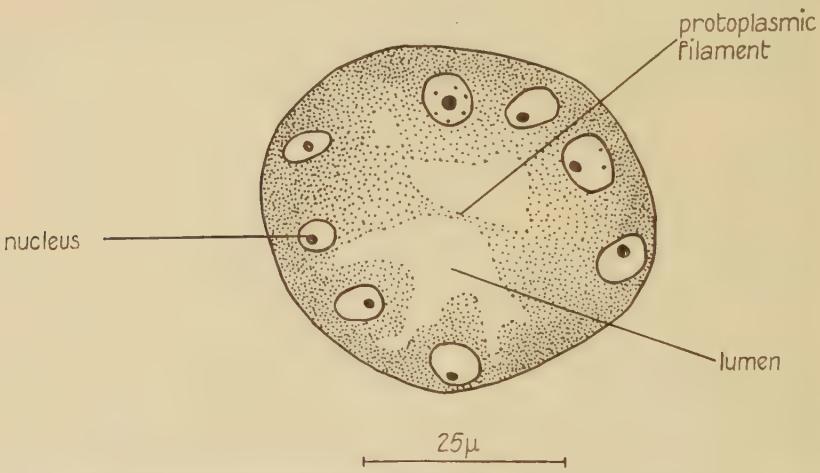

Fig. 3. *D. latreilli*. Post-diverticular mid-gut, middle region, inactive phase. Susa: haemalum/acid fuchsin/metanil yellow/light green.

part of the narrow dorsal limb and all the stercoral pocket, which must be considered as an enlarged portion of it. The papillae are about 20 μ high and blunt. The cytoplasm stains most deeply at the base. Some sections of the stercoral pocket show a definite brush-border, 2·5 μ high, in the resting condition. In both the second and third regions the nuclei lie at the bases of the lobes and papillae. As in the first region some nuclei contain scattered chromatinic material while others contain nucleoli. There are occasional pairs of contiguous nuclei.

It has been pointed out by previous authors that the diverticular epithelium is bounded by a peritoneal epithelium one cell thick which becomes several cells thick around the post-diverticular mid-gut. The cells covering the diverticula are roughly hexagonal and 10–12 μ high, although in places the height is much greater. Each cell contains a single nucleus in the meshes of spongy cytoplasm (fig. 1).

The mid-gut has no intrinsic musculature. Gut movement is apparently carried out by the muscles which stretch from tergite to sternite between the gut diverticula, by general body movement, and possibly by some contractility of the gut epithelium.

Protein Digestion

As both the excretory and digestive cells contain at some stage discrete intracellular protein globules, the fate of these globules in the two types of cell has been closely followed. An account of the changes occurring in the digestive cells is given below, followed by a description of the developmental sequence in the excretory cells.

The food entering the mid-gut is wholly liquid, all solid particles having been removed by a preoral filter (Vachon, 1934). The food 'Brei' enters all the diverticula and bathes the tops of the epithelial cells. This Brei, which stains green by the quadruple method, gives a positive reaction for the amino-acid, arginine. Shortly after the beginning of feeding, e.g. after $\frac{1}{2}$ hour, the digestive cells previously empty after 12 days' starvation, contain apical inclusions staining with light green and giving a positive reaction for arginine. These inclusions are of various sizes, the largest being of about 17·5 μ diameter. It is believed that these inclusions are composed of protein or of protein-derived material absorbed from the lumen of the gut. The formation of these globules continues while there is Brei in the lumen until the cell is full.

Five hours after the beginning of feeding, although the inclusions still contain arginine, the staining reaction of many of them has altered. Apical inclusions still stain green by the quadruple method but those in the rest of the cell, presumably those absorbed earlier, stain with either metanil yellow or acid fuchsin, or show combinations of stains, yellow-green or red-yellow (fig. 4). Accompanying this change the inclusions become smaller and more compact; in one animal 5 hours after the beginning of feeding, the green inclusions measured up to 17 μ diameter, the red and yellow up to 12 μ.

This state in which the cells contain inclusions showing the complete range of staining reaction is seen as late as 24 hours after the beginning of feeding. However, by 40 hours the green inclusions have disappeared and all inclusions stain red, yellow, or yellow-red. Some animals at 40 hours show nothing but acid fuchsinophile inclusions in their digestive cells, and by 4 days all animals appear to have reached this stage. Some time after this, at about 5 days, the fuchsinophile inclusions, which have hitherto been scattered throughout the cell, come to lie apically. Here they undergo a marked change which may be

seen affecting some cells as early as 5 days and which is widespread at 7 days. The staining reaction of the inclusions by the quadruple method changes from a brilliant red to a dull chocolate or reddish purple. At the same time the inclusions become granular in appearance. Simultaneously they lose their arginine-positive reaction (fig. 5).

Between a third and a quarter of the cell is then constricted from the free end and shed into the lumen, carrying with it the undigested granular material

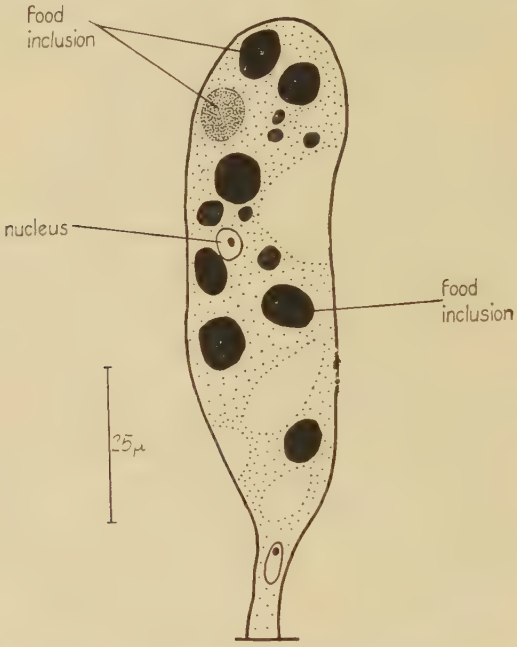

FIG. 4. *D. latreilli.* Digestive cell 14 hours after feeding, showing one green staining inclusion (stippled) and several yellow or red staining inclusions (black). Susa: haemalum/acid fuchsin/metanil yellow/light green.

and often a nucleus. The spheres of undigested granular material usually break up into smaller granules of about 2 μ diameter.

As early as 5 hours after the beginning of feeding some of the green- and yellow-staining inclusions in the digestive cells can be seen to be darkening and becoming granular; they appear to break down into what Frank (1937) has called 'cell faeces' without the inclusions going through an acid fuchsinophile stage. The formation of faecal material in the digestive cells is mainly by way of the fuchsinophile inclusions. The other method appears to be subsidiary to this.

Excretory Cell Development

At any one time the number of developmental stages of the excretory cells present in the diverticular mid-gut is limited. There are often several stages

represented but one does not find the whole developmental series present in one animal at one time. Instead, large numbers of cells occur showing a few stages. In the earliest stage at which these cells are recognizable they contain no inclusions; they are small, 25 μ or less in height, about 10 μ broad, and filled with a dense cytoplasm staining green by the quadruple method. At this stage small inclusions, about 2 μ diameter and staining with metanil yellow, appear in the cells. These inclusions grow in size and number until there are between 30 and 60 present in each cell. During the increase in size

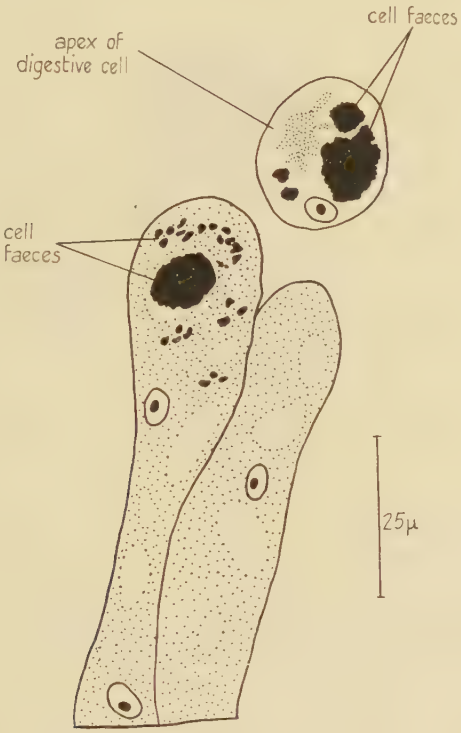

FIG. 5. *D. latreilli.* Two digestive cells 7 days after feeding. One contains apical cell faeces while the other has shed its apex. Susa: haemalum/acid fuchsin/metanil yellow/light green.

the staining reaction of the globules alters and they stain brilliantly with acid fuchsin (fig. 1). At both the metanil yellow stage and the acid fuchsin stage they show a positive reaction for arginine.

The appearance of the globules now alters greatly. They are transformed into opaque granules varying in size between 1 and 5 μ in diameter (fig. 6). This change affects globules of all sizes. The granules, like the globules, stain with acid fuchsin but now show a wine colour. This change in the staining reaction is paralleled with the iron haematoxylin method. The globules stain faintly yellow if at all, while the granules stain a deep blue. The mass of granules in the cell has the appearance of a morula and at this stage it is shed into the

lumen. The granules at this point contain arginine. The shedding often entails the release of the whole cell, the nucleus being situated in a thin rind of cytoplasm persisting round the granular mass. The appearance at this stage supports the contention that the cell inclusions are situated in a vacuole. At times it seems that the nucleus and some of the basal cytoplasm are left *in situ* when the morular mass is shed. On reaching the lumen the cell loses its

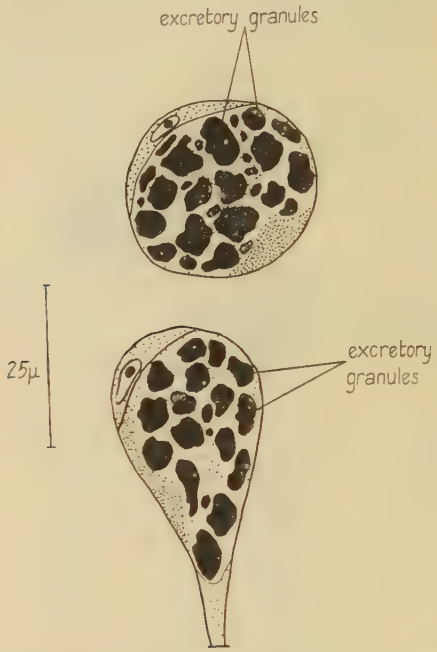

FIG. 6. *D. latreilli*. Two excretory cells containing excretory granules. One is attached to the diverticular wall, the other extruded and free in the gut lumen. Susa: haemalum/acid fuchsin/metanil yellow/light green.

elliptical form and becomes spherical, the spheres varying in size from 20 to 40 μ in diameter.

Some excretory cells do not show a morula stage. In these the fuchsin-staining globules are transformed directly into an amorphous mass appearing grey in section. This material gives a negative reaction to Sakaguchi's test. It is subsequently shed into the gut lumen. The two types of excretory cell development occur together in the same animal and both sequences are equally numerous.

There seems to be no direct relationship between the activity of the digestive cells and that of the excretory cells. After prolonged starvation, of a month or more, small rhomboidal crystals with sides about 1 μ long occur between the globules of the excretory cells. The origin and fate of these crystals has not been studied but when they are present in the diverticular gut they are also present in the lumen of the post-diverticular gut.

The Activity of the Post-diverticular Mid-gut

Although three histologically different regions of the post-diverticular ntestine have been described, at least part of the activity of these regions is he same, the differences being matters of degree rather than of kind.

The morulae formed in the diverticula by the excretory cells pass along he post-diverticular mid-gut and in so doing they are taken up by the wall of the tube, including that of the stercoral pocket (fig. 7). This process is least

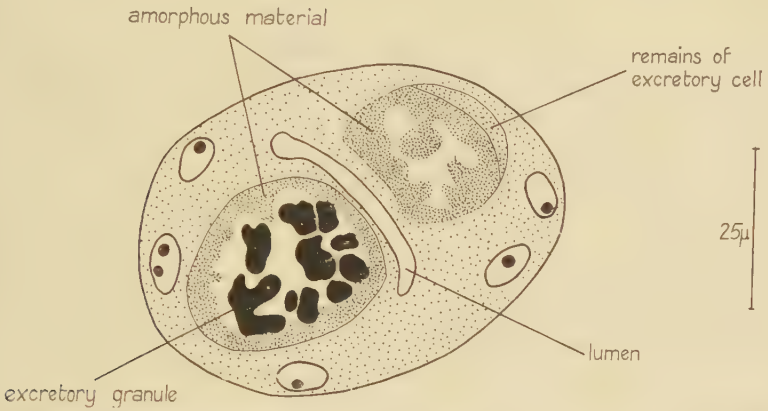

Fig. 7. *D. latreilli*. Post-diverticular mid-gut, middle region, active phase. Two extruded excretory cells in cytoplasm, one of which is completely converted into amorphous material. Susa: haemalum/acid fuchsin/metanil yellow/light green.

frequent in the ventral limb. Although morulae embedded in the wall of the ube are frequent in sections, how the uptake occurs is not known. In the epithelial cytoplasm the morulae are transformed into amorphous, opaque, non-staining masses which, unlike the parent material, give negative results with Sakaguchi's test for arginine. These opaque masses resemble in appearance the material formed in the excretory cells without the cells passing through a morular stage. Simultaneously with the conversion of the morulae nto amorphous material the epithelium appears to secrete a similar material nto the lumen. This activity causes a considerable breakdown of the cytoplasm which is most pronounced in the stercoral pocket and in the papillate egion of the dorsal limb, and least pronounced in the smooth-walled ventral imb. The height of the epithelium in all regions is reduced during the passage of the morulae but least in the ventral limb.

The opaque material collects in the stercoral pocket and is there changed nto a mass of minute rhomboidal crystals with sides about 1 μ long. The faeces on extrusion consist of pellets of this crystalline material together with cellular debris. The solubility of these crystals indicates that they are possibly purine, and if a purine, that they are guanine (see Lison, 1936). The crystals re soluble in sodium hydroxide solution, potassium hydroxide solution, and n nitric, hydrochloric, and sulphuric acids. They are insoluble in water,

ethyl alcohol, xylene, ammonia solution, acetic acid solution, and in an aqueous solution of piperazine. Insolubility in the last solvent shows that they are probably not uric acid. Murexide tests with both nitric acid and chlorine gas as oxidizing agents were carried out on the faecal material but the results were inconclusive.

The activity of the post-diverticular mid-gut during the passage of 'cell faeces' from the digestive cells was obscured in all sections examined by the presence of excretory morulae in this region of the gut.

Food Storage in the Mid-gut

The false scorpion material was tested for protein (by Sakaguchi's test), glycogen, and fatty material. Of these substances only glycogen and fat are stored; protein globules are present in the mid-gut only at certain stages in the digestive and excretory processes which are described above. The distribution of glycogen and fat are described below.

Both *Dactylochelifer latreilli* and *Neobisium muscorum* were used in the tests for glycogen; no differences between the two animals were observed. The main site of glycogen storage is in the peritoneal epithelium, especially in the tissue around the post-diverticular mid-gut. In this region the cells of well-fed animals are so full of glycogen as to obscure the cell boundaries in Bauer-stained material. The glycogen is distributed in the cytoplasm in the form of granules.

The digestive cells of the mid-gut appear to be a site of subsidiary storage, glycogen being present in them and in the peritoneal tissue even after 2 or 3 months' starvation. The glycogen is here distributed throughout the cells except at the apices. As in the peritoneal cells it is present in the form of granules. The presence of glycogen in the digestive and peritoneal cells was shown by both Bauer's and the iodine method.

Glycogen was demonstrated in small quantities in other regions by Bauer's method. The quantities were too small to be detected by the iodine technique. These sites were: between the protein globules of the excretory cells, small areas in the active epithelium of the post-diverticular mid-gut, and in some faecal material in the post-diverticular mid-gut.

The distribution of fatty material was investigated in *D. latreilli* and in *N. muscorum*. As was mentioned before, the digestive cells and the peritoneal cells are sites of storage. The digestive cells are especially rich in sudanophile material which is scattered throughout them as spherical droplets varying in size from 2·5 to 10 μ. The food Brei is very rich in sudanophile material; so probably at some stages some fat in the digestive cells is not being stored but is merely passing through the cells. However, after one month's starvation osmiophile globules of the same size as the fat globules were found in these cells. It thus appears that the cells are sites of prolonged storage.

The fat in the peritoneal cells always presents the same appearance. It is evenly scattered in the cytoplasm in the form of small globules of about 4 μ diameter.

In frozen sections of one specimen of *N. muscorum* the globules in some excretory cells were markedly sudanophile. This is the only occasion on which large quantities of fatty material were found in the excretory cells.

In the post-diverticular mid-gut the amount of fat present is very small, and is represented by sudanophile globules, of diameter 1–2 μ, scattered throughout the epithelium of all regions. No material showing positive coloration was found in the faeces.

Discussion

The diverticular mid-gut of Chelonethi like that of the Arachnida in general is composed of a number of types of cell. However, while in the Arachnida as a whole the two main types of cell are claimed to be digestive and enzyme-secreting cells respectively (Millot, 1949), those of the Chelonethi investigated are digestive and excretory. Bruntz (1904) found that certain cells in the diverticular epithelium of false scorpions excreted injected dyes but that such cells were absent in scorpions, spiders, and harvestmen. Although the conclusions that may be drawn from such experiments are doubtful, it is relevant to note that in the latter three orders the diverticular mid-gut is claimed by other authors (Millot, 1926; Pavlovsky and Zarin, 1926; Frank, 1937) to be composed mainly of digestive and secretory cells. Furthermore, Malpighian tubules are present in these groups while they are absent in the Chelonethi. A mid-gut possessing both excretory and digestive cells, as in the Chelonethi, is described by Fretter (1939) for certain tectibranch molluscs.

Although the diverticular region of the gut epithelium is not typical, the digestive cells of the false scorpion gut seem to function in a way similar to those of other intracellularly digesting animals. Protein or protein-derived material is taken into the cell and undigested material is later shed into the gut lumen. The time needed for this digestion to take place varies in different Arachnids; in *D. latreilli* the 'cell faeces' were voided after 7–8 days. A similar time has been found to elapse in spiders (Millot, 1926). Frank (1937) found that in harvestmen the 'cell faeces' were voided after 24 hours.

Although the activity of the ferment cells in spiders is directly correlated with feeding while the activity of the excretory cells in false scorpions is not, the products of the cells are in some respects similar. Millot (1926) states that the spheres of the ferment cells, which stain markedly with acid fuchsin, are discharged into the gut lumen as a morular mass, a process paralleled in the excretory cells of the false scorpions. The products of the excretory cells and of the ferment cells are presumably both of internal origin unlike the 'cell faeces' shed from the adjacent digestive cells.

The presence of replacing cells in the diverticular mid-gut of false scorpions is to be expected from the type of activity described for the excretory and digestive cells, and from the general statements of Schlottke (1934) and Yonge (1937) on intracellular digestion. Whether the cells lying between the bases of the other cells fulfil this function is unknown.

The other Arachnid order besides the Chelonethi possessing a long post-diverticular mid-gut is the Scorpiones (Pavlovsky and Zarin, 1926). It is not known whether the physiology of this region in scorpions resembles that in false scorpions. The excretion in the mite *Tyroglyphus farinae* (Hughes, 1950) is similar in some respects to the excretion from the post-diverticular mid-gut of *D. latreilli*. In *Tyroglyphus* the colon, a region comparable with the post-diverticular intestine of the false scorpion, secretes into the lumen a liquid from which guanine crystallizes. Like false scorpions, *T. farinae* has no Malpighian tubules. The activity of the Malpighian tubules of spiders as described by Millot (1926) also resembles that of the post-diverticular mid-gut of false scorpions in that a pre-guanic material secreted by the syncytial epithelium crystallizes in the lumen. In neither spiders nor *T. farinae* is there anything comparable with the uptake of morulae found in false scorpions. The stercoral pocket of *D. latreilli* is very similar to that of spiders in being syncytial, papillate, and in having a brush-border. In spiders the pocket is said to be an enlarged portion of the Malpighian tubule system, while in false scorpions it is undoubtedly an enlargement of the narrow post-diverticular mid-gut. In both groups the pocket has an excretory function as well as storing faeces.

Glycogen and fatty material are important storage products in false scorpions, both being stored in the digestive cells and in the peritoneal cells of the mid-gut. In other Arachnida information about the distribution of glycogen is scanty. Millot (1926) failed to find it in spiders; in *Tyroglyphus* (Hughes, 1950) it is the main storage product, being stored in the tissue surrounding the gut. In spiders (Millot, 1926) and in harvestmen (Frank, 1937) fat is the main substance stored, being found in quantity in the digestive and peritoneal cells, as in false scorpions. With the techniques used in the present studies on false scorpions it is difficult to evaluate the relative importance as storage materials of glycogen and fat.

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REFERENCES

Baker, J. R., 1947. Quart. J. micr. Sci., **88**, 115.
Bensley, C. M., 1939. Stain Tech., **14**, 47.
Bernard, H. M., 1893. J. Roy. Micr. Soc., **13**, 427.
Bertkau P., 1887. Verh. nat. Vereins der preussischen Rheinlande usw., **44**, 112 (original not seen).
Bruntz, L., 1904. Arch. Biol., Paris, **20**, 217.
Croneberg, A., 1888. Bull. Soc. Nat. Mosc., **2**, 416.
Frank, H., 1937. Z. Morph. Ökol. Tiere, **33**, 151.
Fretter, V., 1939. Trans. R. Soc. Edin., **59**, 599.
Hughes, T. E., 1950. Quart. J. micr. Sci., **91**, 45.
Kästner, A., 1927. *Pseudoscorpiones* in Schulze's *Biologie der Tiere Deutschlands*, Lief. 25, T. **18**. Berlin.
Lee, A. Bolles, 1937. *Microtomist's Vade Mecum*. 10th ed. London.
Lison, L., 1936. *Histochimie animale*. Paris (Gauthier-Villars).

McIntire, S. J., 1868. J. Quekett Micr. Cl., **1,** 69.
Millot, J., 1926. Bull. biol. Fr. Belg., Supplement **8,** 1.
—— 1949. *Traité de Zoologie*, **6**, 263. Paris.
Pantin, C. F. A., 1948. *Notes on microscopical technique for zoologists.* Cambridge.
Pavlovsky, E. N., and E. J. Zarin, 1926. Quart. J. micr. Sci., **70,** 221.
Roewer, C. F., 1937. *Chelonethi oder Pseudoskorpione.* Bronn's *Klassen und Ordnungen des Tierreichs.* Leipzig.
Rossmann, I., 1940. Amer. J. Anat., **66,** 227.
Schlottke, E., 1934. Biol. Zbl., **54,** 51.
Serra, J. A., 1946. Stain Tech., **21,** 5.
Vachon, M., 1934. Bull. sci. Bourgogne, **4,** 38.
Yonge, C. M., 1937. Biol. Rev., **12,** 87.

The Formation of the Ootheca by *Periplaneta americana*

II. The Structure and Function of the Left Colleterial Gland

By P. C. J. BRUNET

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With two plates

Summary

The anatomy of the left colleterial gland of the cockroach is described. It is convenient to subdivide the gland into four regions. At the posterior end, abutting on to the outlet of the gland, there is no secretory activity and the cells of this region are not unlike normal epidermal cells. Anterior to this region are the three secretory regions of the gland; of these, the anterior and posterior secrete the structural protein and the constituent cells are equipped with a complex end-apparatus, a thick-walled depression in the apex of the cell in which the final stages of the elaboration of the secretion occur. The body of the end-apparatus contains canalicules which lead to the lumen of the gland. Alkaline phosphatase is abundantly present in this organ. Protein is secreted continuously, and there is no cycle related to oviposition. The third region, between the protein-secreting regions, secretes an oxidase, whose function may well be to oxidize the phenolic tannin-precursor produced in the right colleterial gland when the products of the two glands come together at oviposition.

The gland becomes fully functional some 14 days after the final moult, the immature cells of the nymph developing directly into mature cells characteristic of the region in which they occur. Within each of the main regions the cells show some differences which suggest that there is a wave of change passing along the gland. It appears that the cells of the anterior end of the gland become defunct and the hitherto inactive cells of the posterior end take on a secretory function.

The structural protein has a high phenolic content, and contains no combined carbohydrate. The presence of lipoid in the secretory cells appears to be directly bound up with the secretory processes.

Introduction

THE colleterial glands of the cockroach are the sexual accessory glands which produce most of the material used in the formation of the ootheca, the case which serves to protect each batch of eggs that is laid. The fabric of the ootheca and the substances which harden the arthropod cuticle are biochemically similar (Pryor, 1940*b*), and this gives to the results of the study of the colleterial glands a broad applicability and a more than parochial significance. The microanatomy and location of these glands has been described by Bordas (1909), Ito (1924), Voy (1949), and Brunet (1951). The glands are made up of a right- and left-hand component; each consists of a mass of branched tubules lying freely in the haemocoel, and each has its own opening into the genital vestibulum, an invagination of the posterior end of

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the abdomen concerned with the bringing together of eggs, stored sperms and other materials used to complete the formation of the ootheca and its contents.

In macroscopic appearance the left and right gland differ from one another. The left gland is composed of more tubules, and is therefore larger than the right, which it more or less surrounds. The colour of the left gland is mostly whitish on account of the presence of secretion product in the lumen, while the right gland stores little secretion in the lumen and is translucent and colourless. The function of the left gland is to secrete a protein which becomes shaped to form the ootheca (Pryor, 1940*a*) and the right gland secretes a diphenolic substance (Pryor, Russell, and Todd, 1946), which is converted by enzyme action to a quinonoid tanning agent that serves to cross-link protein molecules. The resulting tanned protein of the ootheca has a particularly resilient nature.

The aim of this paper is further to describe the anatomy of the secretory cells of the glands, and where possible to use histochemical methods to infer the part played by them in the production of the ootheca.

Material and Methods

Periplaneta americana has been used almost entirely for the investigation. This cockroach is easily cultured at a temperature of 28° C., which is favourable to the insect. The glands of *Blatta orientalis* have also been examined. There is a very close similarity between the colleterial glands of *Blatta* and *Periplaneta.*

Anatomical methods. It is possible to remove and examine each colleterial gland separately, in order to distinguish stages of the left gland from those of the right, and this method was used at first. The statement that the left gland appears whitish (since the lumen is filled with opalescent protein) while the right gland appears translucent (Ito, 1924; Pryor, 1940*a*) was used as a guide when disentangling the glands. However, it is only a general rule; for those cells which I have classified as type 3 cells of the left gland (Brunet, 1951) secrete little or no opalescent protein and the tubules in which they occur thus appear translucent, like the tubules of the right gland. Since also these same type 3 cells are taller than the remainder of the cells of the left gland, being approximately the same size as the cells of the right gland, unqualified acceptance of this method of distinction can be misleading. The tubules are entwined and bound together by tough and almost invisible tracheae; it is difficult to separate them without causing damage. It has been found preferable to deal with the glands while still attached at their exits to the dorsal wall of the vestibulum. Two longitudinal cuts along the edge of the full length of the abdomen allow the terga to be lifted up, and if these cuts are continued at right angles so as to meet below the anus, the terga and alimentary system are then made free and can be shifted anteriorly out of the way. The whole reproductive system and accessory glands are then clearly visible, and can be removed and examined or fixed and sectioned serially.

Histological and cytological methods. Fixation in Bouin's fluid, followed by staining with Ehrlich's haematoxylin and eosin proved to be an adequate general technique. Meves's modification of Flemming's chromic/acetic/osmium fixative (see Baker, 1945) followed by Heidenhain's haematoxylin was used for general study of the cytoplasm and of the end-apparatus of the secretory cells. Altmann's dichromate/osmium fluid was used as a non-precipitant fixative, and was likewise followed by Heidenhain's haematoxylin. Many sections of tissue fixed in neutral formaldehyde/saline (Baker, 1949), and intended as controls for histochemical work, were used for the interpretation of general anatomy. As a basic dye 0·1 per cent. aqueous solution of toluidine blue G was often used in place of dye lakes; staining time is very short, and, as a brilliant blue counterstain, it is often useful in conjunction with histochemical techniques, for which purpose its transparency is an advantage.

For mitochondria, material was fixed in Helly's dichromate/mercuric chloride/formaldehyde and post-chromed for 48 hours at 37° C. in saturated potassium dichromate solution. Altmann's acid fuchsin and other triphenylmethane dyes dissolved in aniline water were used for colouring the mitochondria, and this treatment was variously followed by picric acid or aurantia (see Baker, 1933 and 1945), or methyl blue (Cain, 1948), or methylene blue derivatives (Volkonsky, 1928). Owing to its intensity, acid fuchsin used thus proved to be an excellent routine acid dye.

To determine whether fixing mixtures, known to be powerful evocators of myelin-forms (Palade and Claude, 1949), would show up the classical Golgi network, Flemming's fluid was used, followed by post-osmication for several days at 37° C., and Aoyama's formaldehyde/cadmium fixing mixture was used, followed by treatment with silver nitrate and subsequent reduction. This last fixative acted brutally upon the secretory cells, causing them, in many cases, to eject the secretory end-apparatus (an inpushing at the apex of the cell, larger than the nucleus).

Baker's method (1949) using formaldehyde/dichromate for fixation followed by post-chroming and colouring with sudan black was used for demonstrating lipochondria, a term used by Ries (1935), Holtfreter (1946*a*), and Baker (1950), and used here in the last-named author's sense to denote the lipoid granules and the lipoid sheaths customarily found to be present around secretory materials within a cell. The term lipochondria has recently been adopted by Baker (1950) to denote these organelles as a result of their dissimilarity to the original apparatus of Golgi. The term is equivalent to the Golgi bodies (of Baker, 1949); Golgi system (of Hirsch, 1948), and spheroids (of Thomas, 1948).

Except for this last technique, paraffin embedding was used. The presence of the cuticle does not seriously upset section-cutting. In the neighbourhood of the cockroach's vestibulum there is not a great deal of heavily sclerotized cuticle, and the surfaces are sufficiently contorted to allow firm interlocking of wax and cuticle. Sections fixed for colouring with Baker's sudan black were cut from frozen gelatine blocks. These and other sections for histochemical

purposes cut from gelatine blocks show that this is a most admirable method for cutting sections of cuticular tissues. There are limitations to the method: for example, the boundaries of cells or cuticle lying against supporting gelatine are often hard to define since the gelatine itself is coloured by dyes. Use was made of formaldehyde/alum solution (Baker, 1949) instead of neutral formaldehyde for hardening the gelatine blocks. Sections cut from blocks so hardened have the great advantage over formaldehyde-hardened blocks that they do not tend to stick to vessels and instruments.

Observations of living tissue. 0·01 per cent. solutions of various basic dyes in 0·7 per cent. saline (Baker, 1951) were used to demonstrate cell organelles. Since these so-called vital dyes are sometimes known to alter the nature of the constituents of the cell (Holtfreter, 1946*b*), unstained living cells were also examined by phase-contrast microscopy.

Histochemical methods. A number of qualitative colorimetric, mainly *in situ*, methods were used, which are discussed below in the text.

Observations

The Secretory Stages of the Left Gland

The four main types of constituent cell have been described (Brunet, 1951). The method of subdivision is arbitrary, and is such that any one of the four types of cell is quite unlike any other type (cf. figs. 1, 4, 12, and 18); and there occurs, between the region occupied by any one type of cell and its neighbour, a region of rapid change in cell character. The type 1 cell is found at the posterior end of the gland; it is not secretory and resembles an epidermal cell. The type 2 cell is a squat cell, much of the volume of which is occupied by the end-apparatus (fig. 4); a dense protein secretion is secreted in this region. The type 3 cell is a tall cell with an elongated end-apparatus, and with clearly fibrillated cytoplasm basally (fig. 12). The type 4 cell forms most of the gland and is a short cell with a more or less spherical end-apparatus out of which protein passes into the lumen of the gland (fig. 18). There is no cyclical change occurring in the cells in relation to the formation of the oothecae: it is quite clear in types 2 and 4 cells—the two regions that secrete structural protein—that the lumen of the end-apparatus is always filled with protein, and there is no change in the cytoplasm indicative of such a secretory cycle. Protein is continually being produced and poured into the lumen of the gland.

The colleterial glands are present in the last instar nymph in an undifferentiated condition and the cells of both left and right glands are extremely similar, and are like cells of the epidermis. During the first day of the adult instar, the tubules of the left gland begin to show differentiation into the four definitive types of cell, but there is no secreted protein in the lumen of the tubules. On the second day of the adult instar, the four cell types are easily distinguishable and there is a trace of secretion in the lumen; and by the seventh day there has been a great increase in the size of the gland by growth on the

art of the cells, and in particular of their cytoplasm; by this time there is ıore protein in the lumen. By the fourteenth day the gland is mature and viposition commences.

The disposition of the cells within the mature gland is not straightforward. ıs has been described, a tubule is composed of four types of cell arranged in near sequence, and each type is separated by a transitional region, where ıere is a *rapid* but graded change in cell character. There is also, however, *slight* change in cell character within the region occupied by any one cell ype; for example, type 2 cells at the height of their activity are more or less quidimensional (fig. 1), but there is a slight progressive increase in height f the cells towards the anterior limit of the type 2 cell region until the transi- onal region between types 2 and 3 cells occurs and where the increase in eight is rapid. Type 3 cells then show a further steady increase in height ollowed by a slight decrease, until the transitional region between types 3 nd 4 cells occurs, where the decrease in height is rapid. Type 4 cells near the ransitional region are relatively tall, but there is a steady decrease in height until ıe condition where all the cells are more or less equidimensional is reached.

The significance of this arrangement is not at once evident. It is clearly not hat would be expected if the glandular cells were passing through a mono- hasic secretory cycle such that types 2, 3, and 4 cells represented early, iiddle, and late stages of secretion. In the first place, the well-defined transi- onal regions are not compatible with such an hypothesis, and also since in ntogeny all the secretory types differentiate out at the same time (i.e. on the ourteenth day of the adult instar). The presence of mitochondria and lipo- hondria in all three cell types suggests that they are all active secretory cells, nd histochemical evidence shows that they are secreting not the same but ıree different products. This being so, the presence of three regions of secre- ory cells and the intermediate transitional regions is understandable, but the eady change within the regions occupied by the secretory cells still requires ı explanation. It is tentatively suggested that there is a slow wave of change assing along the tubules of the gland such that as time passes each cell takes ı the function previously carried out by a cell situated anteriorly to it. As a esult of this, the most anterior type 1 cells become secretory (indeed, the anterior ype 1 cells show first a dense apical region of the cytoplasm and later a idimentary end-apparatus which is most clearly defined in the most anteri- rly situated cells). In time these cells are thought to become fully secretory ype 2 cells. These latter are destined to become type 3 cells, which themselves ecome type 4 cells; and as this process occurs the most anterior type become educed in size and are evidently effete. The hypothesis is represented dia- rammatically in Table 1. The reason as to why this process should occur is bscure. Table 1, being diagrammatic, of necessity suggests the presence of latively more transitional cells than in fact exist. This being so, it can be en that the number of cells which actually change from one cell type to ıother during the life-history is small, and what value such a slow wave of ıange can possess for the organism is not understood.

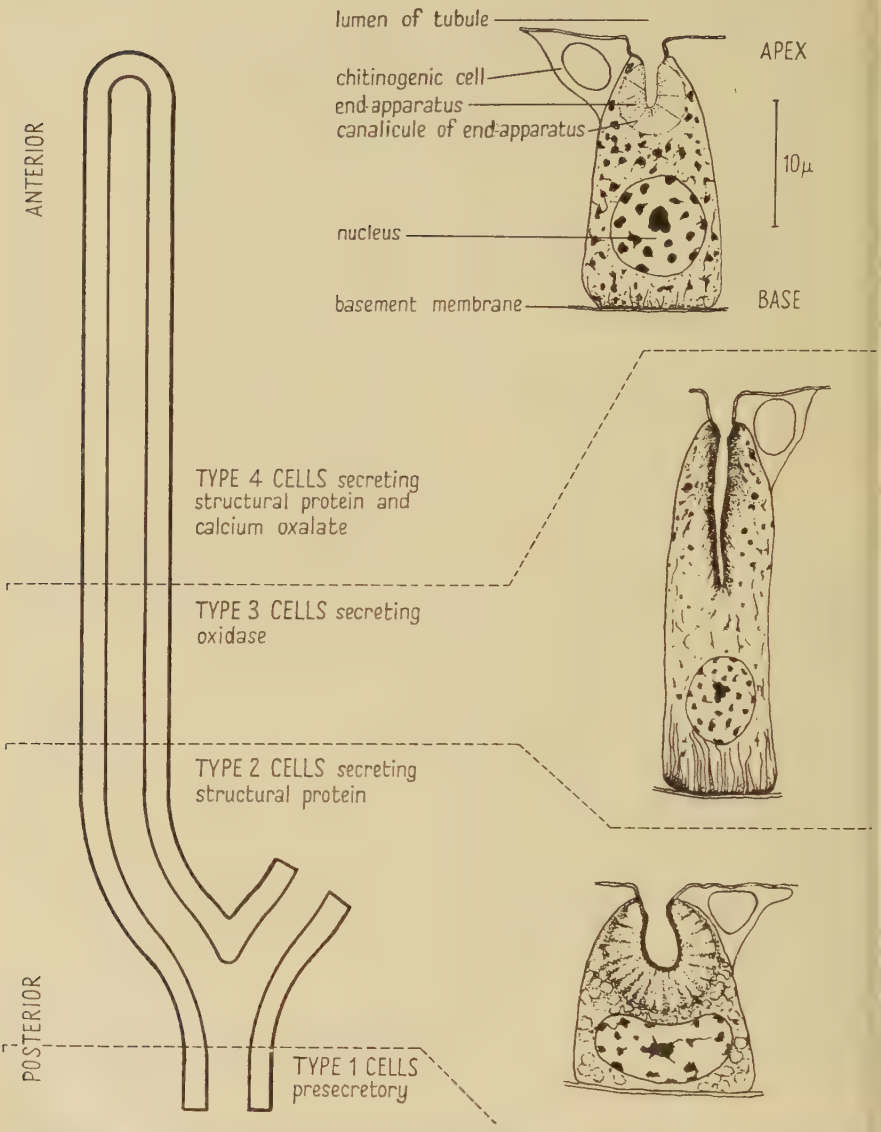

FIG. 1. The secretory cells of the left colleterial gland as they appear after staining with iron haematoxylin. Their approximate position in the gland is indicated. All but one branch of the gland has been omitted for the sake of clarity.

TABLE 1

	Posterior end of gland																							Anterior end of gland
ıst nymphal instar	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
dult instar 1st day	1	1	1	1	1	1-½	2	2	2	2	2	2	⅔	3	3	¾	4	4	4	4	4	4	4	4
dult instar 7th day	1	1	1	1	1	½	2	2	2	2	2	2	⅔	3	3	¾	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*
dult instar 14th day t oviposition	1	1	1	1	1	½	*2*	*2*	*2*	*2*	*2*	*2*	⅔	*3*	*3*	¾	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*
d oviposition	1	1	1	1	½	*2*	*2*	*2*	*2*	*2*	*2*	⅔	*3*	*3*	¾	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*
d oviposition	1	1	1	½	*2*	*2*	*2*	*2*	*2*	*2*	⅔	*3*	*3*	¾	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*	*4*

The table illustrates the author's hypothesis as to the ontogeny of the four cell types in the ft colleterial gland. The types of cell constituting the gland are indicated by numerals, and e table shows the development from the unspecialized nymphal condition to the adult ndition, and the slow shift of function during the adult condition.

Arabic numerals indicate the types of cell present at any one period; two small numerals dicate transitional types; italic numerals indicate that the cells are pouring secretion out to the lumen.

The number of cells of any one type is *only approximately indicated* by the number of ımerals, e.g. type 3 cells are in the minority, and type 4 cells in the majority.

he Nature of the Structural Protein.

Structural protein is secreted by two distinct regions of the left gland; cells f type 2 (fig. 4) and type 4 (fig. 18) carry out this function. Both types of cell ave certain similarities in that they are short, squat cells with a somewhat ounded end-apparatus, and they contrast with the type 3 cells, which are tall ells with an elongated end-apparatus. It is not known why there should be two eparate secretory regions; there are two also in the silk glands of *Cossus* and ther silk-spinning larvae (Bradfield, 1951), and in this case it is believed that ach secretes one of the two components of silk.

In *Periplaneta* large characteristic crystals of calcium oxalate (Kadyi, 1879) re mixed with the abundant protein secreted by the type 4 cells. These do ot occur in the more scanty protein secreted by the type 2 cells. After rmaldehyde fixation type 2 protein appears to be more coarsely granular han type 4. This difference is more evident in watery mounts than when the ssue is in balsam.

With *in situ* histochemical tests, whether on fresh material or paraffin ections, no distinction can be drawn between the two types of protein; but his does not necessarily indicate close similarity. Histochemical methods are ot very suitable for protein analysis: many are exceedingly brutal in action, nd many show simply an organic configuration (not that of an α-amino acid) om which one infers the presence of a protein. If an unknown substance acts positively to several of these tests, there is considerable likelihood that contains or consists of protein, and this likelihood is increased if a positive action is given with a reagent that demonstrates the presence of the peptic nkage or of amino-acid.

Ninhydrin in 50 per cent. glycerol, and alloxane 1 per cent. in 70 per cent. cohol (Lison, 1936) both gave intensely positive reactions with the protein

in the lumen, thus indicating an amino-acid configuration. These reactio only occur with fresh material. Only a very weak reaction occurs after fo maldehyde fixation, presumably as a result of the reaction of formaldehy with the amino-group concerned in the test.

Baker's (1947*a*) modification of Sakaguchi's test showed the presence arginine or a related guanidine derivative. The colour produced was n intense.

An intensely positive xanthoproteic reaction implied, and a similar reacti with Millon's reagent demonstrated, the presence of a phenolic nucleus. T result of coupling with several diazo-compounds in alkaline solution, in t manner specified by Lison, was to produce a coloured compound, and wou thus further indicate the presence of a phenolic compound (Lison, 1931 a 1936). The specificity of this test is further considered below. Syrupy pho phoric acid (Romieu, 1925) caused the production of a pale pink colo attributable to the presence of tryptophane.

Oxidation with periodic acid (Hotchkiss, 1948; McManus, 1948) follow by the application of Schiff's reagent gave completely negative results fixed and fresh material. Periodic acid oxidizes β-hydroxy-α-amino acids su as serine (Nicolet and Shinn, 1939) converting them to aldehydes, which c then be demonstrated with Schiff's reagent. The method has been used f the biochemical estimation of such acids, and Lillie (1950) has shown t possibility of demonstrating such hydroxyamino acids by this means.

With the same reagents, the presence of substances containing the α-glyc group would have been shown: periodic acid oxidizes the hydroxyl groups aldehyde groups, which have been shown to be absent. Oxidation with 4 p cent. chromic acid (Bauer, 1933) again gave rise to no aldehyde groups. The two tests therefore demonstrate the absence of unsubstituted polysaccharide A 0·1 per cent. aqueous solution of toluidine blue showed no metachromat coloration of the protein, and demonstrated the absence of sulphate esters high molecular weight (Lison and Fautrez, 1939), which are usually indicati of the presence of mucopolysaccharides. A negative reaction with Molisch α-naphthol reagent confirmed the total absence of polysaccharides.

A characteristic of the protein secretion is its ability to reduce ammonia silver hydroxide solution (Lison, 1936), and Burtner and Lillie's (1949) mod fication of Gomori's (1946) methenamine (= hexamine or hexamethyle tetramine)/silver nitrate solution. Such reactivity indicates the presence a reducing agent, e.g. sulphydryl-, aminophenyl-, dihydroxyphenyl-, aldehydic-group. Lison considers that only the latter three have the pow to reduce silver solutions, and that the amino-groups are inactivated by for aldehyde fixation. Since aldehydes can be shown to be absent from t protein secretion by means of Schiff's reagent, the effect would seem to due to the presence of a dihydroxyphenyl compound. In confirmation of th the chromaffine test is positive: potassium dichromate, iodate, or iodic ac solutions colour the proteins yellow after prolonged treatment. This test said to be highly specific for diphenols (Lison, 1936). Since all these reactio

occur even after fixation in absolute alcohol, the reducing agent is likely to be dopa, which is insoluble in alcohol, and, as an amino-acid, would be bonded to other amino-acids and thus fixed with the protein. The argentaffine reaction is not intense, and the chromaffine reaction particularly weak, but they indicate that there is probably some dopa present in the secretion.

It is noticeable that this reaction does not occur after fixation in Helly's formaldehyde/dichromate/mercuric chloride solution, while it does occur after fixation in a fluid of similar composition but with the mercury salt omitted. This fact, and the observation, unqualified though it be, by Lison (1931) that mercuric chloride should be left out of fixative mixtures if the tissue is later to be analysed for phenols, suggests that the mercury salt may form compounds with phenols, as the result of which they lose their reducing power. If this is so, then the results obtained by Chèvremont and Frederic's (1943) test for sulphydryl groups should be treated with caution. The test involves the location of reducing agents by means of ferric ferricyanide, which is reduced by them to ferric ferrocyanide, both with and without previous incubation in a mercuric chloride solution. Mercuric chloride is known to block sulphydryl-groups, and substances which react positively without incubation and negatively with incubation in the mercuric chloride solution are said to be sulphydryl compounds. It would, however, be unreasonable to persist with the criticism of this technique without further evidence, and also because in the test for sulphydryl-groups mercuric chloride solution is applied for a brief period to fixed sections, while in the present case unfixed or partially fixed tissues are allowed to react with the mercuric chloride for a prolonged period.

Nitroferricyanide stabilized by means of zinc acetate solution (Giroud and Bulliard, 1933) gave a result, when applied to the protein secretion, suggesting that there was little if any sulphydryl content.

It is not altogether impossible that the argentaffine reaction (and also the diazo and chromaffine reaction) are positive as a result of pyrimidine derivatives being present (Jacobson, 1939); and this point will be more fully discussed under the heading of the right colleterial gland.

The protein of both regions 2 and 4 colours intensely with acidic dyes, and markedly so with triphenylmethene dyes in aqueous solution and also in aniline water such as is used in techniques for demonstrating mitochondria. It is coloured, but not intensely, by Heidenhain's haematoxylin and by acid haematein after pyridine extraction (Baker, 1946).

In so far as comparison is possible, the structural protein of the left colleterial gland resembles the proteins of the insect cuticle as described by Trim (1941), who records a characteristically high aromatic amino-acid content, and a low sulphur and carbohydrate content. In one respect the colleterial protein differs from that of the cuticle: Trim records the presence of hydroxyamino-acids in cuticular protein, while there appears to be none in colleterial protein. These differences, however, may be reconcilable since evidence derived from a study of the right colleterial gland would suggest that hydroxyamino-acids are

intermediate products in the formation of the oothecal tanning agent. This being so, it could be expected that such substances would occur in the cuticle, but not in the left colleterial gland, being kept separate (until oviposition) in the right gland.

The Oxidase Secreted by the Left Gland

Pryor (1940*a*) showed that the conversion of the diphenolic precursor to the quinone tanning agent was an enzymatic reaction. Polyphenol oxidases are known to exist (Keilin, 1936) and, as oxidases, to be demonstrable colorimetrically.

If the colleterial glands are immersed in a solution of Nadi reagent an overall blue colour appears in the cells of the glands, indicating either the presence of a powerful oxidizing agent or of an oxidative enzyme system. The ability to effect this reaction is abolished by prior treatment with 0·002 M cyanide solution at pH 7·3, which indicates that the reaction is the result of the action of an oxidative enzyme and not an oxidizing agent. 0·002 M sodium azide solution at pH 7·3 acts as an inhibitor of the cytochrome oxidase system, but leaves polyphenol oxidase unaffected, and it was found that treatment with azide solution before immersion in Nadi reagent greatly lessened the reactivity of most of the glands, and that a region of the left gland which, when dissected out and sectioned, proved to be composed of type 3 cells, retained their activity, and stood out a deep blue colour. From this it is concluded that an enzyme is present, that type 3 cells of the left gland produce it, and that it is most probably the polyphenol oxidase which oxidizes the diphenol produced by the right gland to a quinone when the contents of the left gland and right are mixed during the formation of the ootheca. This separation of enzyme from substrate until oviposition is what might well be expected.

Experiments similar to those carried out with Nadi reagent but with leucobase methylene blue (Roskin and Struve, 1947) gave comparable results.

Dimethyl-paraphenylene diamine alone would give such a blue colour with quinones, if present, but the reaction was negative; nor was the blue colour derived from methylene blue the result of any 'specific affinity' of the tissue for methylene blue, since application of the unreduced dye showed no greater coloration of the type 3 cell region than of any other.

The Cytology of the Left Colleterial Gland

Type 1 cells. These cells occupy the most posterior end of the gland. They show no sign of secretory activity and have no end-apparatus. They form a single layer of basiphil cells with a relatively large nucleus, and lie beneath a thick layer of deeply folded chitin. The most posteriorly situated type 2 cells line the papilla on which the outlet of the gland into the vestibulum is situated, and here the chitinous lining of the duct is adorned with grouped bristles.

The cells change little during the differentiation of the glands which occurs in the first days of the adult instar; their change involves only a diminution

n height as is the case with the cuticular epidermal cells. Within the region f the gland occupied by type 1 cells the lumen becomes extremely narrow, nd, as the elaborated protein secretion passes outwards, it must pass through his constricted region, around which there is an extremely thick layer of nuscle. This constriction could act as a type of spinneret, and the shearing tresses caused to occur in the outflowing protein might bring about physical enaturation.

Type 2 cells. Between type 1 cells and definitive type 2 cells there is a ransitional region in which the cytoplasm of the potential gland-cells stains nore densely and the end-apparatus develops. Reorientation of the cells ccurs: the single-layered epithelium, found where type 1 cells are present, ecomes modified into a double-layered epithelium with chitinogenic cells ying around the lumen, and glandular epithelial cells with their bases upon he basement membrane. As a result of this, for a certain distance along the ubule, disorientation occurs and the cells are unevenly spaced. In addition, he tubule increases in diameter and there is a slight increase in the height of he glandular cells (fig. 2), which are at this stage taller than either type 1 or lefinitive type 2 cells. Since there is no increase in the volume of the cells orresponding with the increase in diameter of the tubule, spaces are left etween neighbouring cells and these can be seen to persist even in the regions f definitive type 2 cells which are secreting protein into the lumen of the tubule.

The definitive type 2 cell is a most distinctive cell, but, despite this fact, t remains only briefly described (Brunet, 1951). It occupies the walls of the ingle, most posterior duct of the gland, the walls of the two tubules formed s the result of the first bifurcation, and the walls of the four ducts formed as he result of the second, though the extent of its location is variable. It is a ell which appears to be more or less cubical in longitudinal section, but with broader base than apex; the end-apparatus is outstandingly large and com-lex (figs. 3, 4, 5). The nucleus is characteristically indented by the end-pparatus (fig. 4), a phenomenon which can be seen in living cells and is not aused by fixation.

A cell stained with Ehrlich's haematoxylin and eosin after fixation in Bouin's fluid preserves its general outline, and the end-apparatus is seen to ave been stained with eosin and the cytoplasm weakly so. The acid dye, owever, is not intense enough clearly to show up the details of the end-pparatus. Heidenhain's haematoxylin after fixation in Meves's fluid shows far nore detail (fig. 4): the cytoplasm appears as a fine reticulum; the interstices eem to have been occupied by substances subsequently leached out during reparation. The end-apparatus colours a deep grey, which can be seen to be nhomogeneous: the grey is the result of the presence of deeply coloured adial striations lying within the body of the end-apparatus. There is evidence hat such striations represent either the content of minute canalicules or the ubstance around such canalicules, which run from the cytoplasm to the avity of the end-apparatus.

There is an outer wall to the end-apparatus, said, in this type of cell, to be

a chitinous inpushing. It is clearly seen in fig. 5 as the uncoloured region b tween the body of the end-apparatus and the mass of secretion in the lum of the end-apparatus. In these cells it is coloured when Burtner and Lillie (1949) methenamine/silver is used after McManus's (1948) periodic ac oxidation, which should demonstrate polysaccharides (fig. 6); however, chit generally and the chitinous intima of the tubules reacts extremely weakly wi this test while the wall of the end-apparatus is deeply coloured, and th indicates that there is some difference between the intima and lining of t end-apparatus. It has been said that chitin is converted to a polyaldehyde the result of oxidation with periodic acid (Hotchkiss, 1948), but I find th this is not so. The chitin of the cockroach gives at most a very weakly positi reaction with periodic acid/Schiff's reagent or periodic acid/silver comple Nor should it be expected to give a positive reaction, for the acetylglucosamin residues in the chitin molecule possess no unsubstituted α-glycol grou according to the generally accepted formula for these residues (Stacey, 194 Acyl-amino derivatives of glycols are stable in the presence of periodic ac (Jackson, 1944). Lillie (1947) specifies only fungal chitin as reacting positive to this test.

Histochemical tests for proteins show that while the cytoplasm reacts pos tively, it contains far less amino-acid than does the elaborated secretion in t lumen.

Tests for lipoids show how different are these cells from the silk-secretin cells of lepidopteran larvae (Bradfield, 1951, quoting Lesperon), where it claimed that little or none is present. Sudan black after fixation in formald hyde/dichromate and post-chroming (Baker, 1949) shows that the cytoplas is packed with lipoidal granules, and that lipoid is present in the end-apparatu which shows a general diffuse coloration with more intense radial striation (fig. 2). Even without post-chroming, after formaldehyde/calcium fixatio these organelles are seen to be strongly sudanophil. Baker's (1946 and 194 test for phospholipines shows the same cytoplasmic granules to be colour intensely and picks out clearly the striae of the end-apparatus (fig. 5). Pho pholipine preparations suggest that uncoloured granules surrounded b phospholipine occur between the nucleus and the end-apparatus, but analys of the result is complicated by the presence of such large quantities of diffu phospholipine.

Acid fuschin colours the elaborated secretion intensely, and, after fixatio in formaldehyde/dichromate and post-chroming, the cytoplasmic granules a most intensely coloured.

With the 'classical' methods for impregnating the Golgi apparatus, no ne work system is found; indeed, the technique does not usually produce th result in invertebrate cells (Palade and Claude, 1949). Post-osmication follow ing fixation in Flemming's fluid shows the cytoplasmic granules to be osmiophil similarly, treatment with silver nitrate with its subsequent reduction b hydroquinone after fixation in Aoyama's fluid shows deposition of silver on th same granules, often incompletely so; the result being that the granules ofte

appear to have argentiophile crescents attached to them. Aoyama's fluid causes the end-apparatus to be extruded from the cell; this demonstrates that this fixative acts brutally until it has been separated into its components by differential diffusion through a tissue block; but the rigidity of the end-apparatus is implied as the result of this distortion.

The type 2 cell is thus a relatively small, highly active cell, synthesizing protein and secreting it. It is not a cell which first partly synthesizes its secretion, stores it, and later releases it (as is the case with some other cells in the colleterial glands). Acid dyes and tests for protein suggest that there is at any one time no great concentration of protein within the cell, most of the cytoplasm of which is occupied by the secretory mechanism of the cell. The evidence suggests that the lipoid granules of the cytoplasm are probably both of mitochondrial and lipochondrial nature. The results of the acid haematein test for phospholipines is evidence for the presence of either or both: phospholipines constitute a considerable amount of mitochondrial substance, and they occur in the Golgi apparatus (Cain, 1947) which represents the lipochondria of Baker (1950), but the intense diffuse staining of the cytoplasm by acid haematein makes morphological differentiation impossible. Baker's (1949) sudan black technique for lipochondria involves post-chroming at 60° C. Mitochondria are usually not shown by this technique: less material is coloured by sudan black than is coloured by acid haematein in the previous test, and the shape of the objects coloured is often crescentic, suggesting their apposition to a chromophobe spherical granule. Acid fuchsin in aniline water after fixation of the tissue in formaldehyde/dichromate with post-chroming colours intensely what appear to be minute granules in the cytoplasm; their number prevents morphological study, but acid fuchsin used under these conditions electively colours mitochondria.

The end-apparatus is evidently the organ wherein the ultimate stages of protein synthesis occur. The staining reactions alter at this barrier, and alkaline phosphatase activity is centred within the end-apparatus; there is an over-all positive reaction to Gomori's technique (1939), used within the suitable limits prescribed by Danielli (1946), with a slight increase at the cytoplasm end-apparatus interface, and a strong increase at the outer wall (fig. 3), the canalicules appear more positive than the interstices. The role of alkaline phosphatase in fibrous protein secretion is not yet fully worked out, but the presence of the enzyme in regions where such substances are formed is characteristic (Bradfield, 1951). The nature of the phospholipine molecule indicates that it is a potentially highly active substance, and its presence in the radial canalicules is in harmony with the theory that these are the paths through which the secretion passes from the cytoplasm to the lumen of the gland. The fact that conglomerations of refringent material of sudanophil nature, and reacting positively in the acid haematein test are present on the walls of the lumen (fig. 5) and that they appear not to mix with the protein secretion favours the theory that the phospholipines in the end-apparatus are playing a role in the transport mechanism across the end-apparatus.

Type 3 cells. Rapid changes in the dimensions of the glandular cells occur over a distance of about 300 μ, and the height of the cells increases from 15 μ to as much as 50 μ. Accompanying this change, there is considerable modification of the rest of the cell. The end-apparatus of type 3 cells is altogether different from that of the previous type; fig. 6 shows the small cup-like wall of the end-apparatus of a type 2 cell; fig. 7 shows that there is a basal prolongation which extends (figs. 8, 9, 10) until the total length of the end-apparatus is more than three times its original length, and simultaneously the original cup disappears. The same figures show, but less clearly, that the main body of the apparatus is also modified; figs. 6 and 7 show the body as a pale striated region surrounding the outer wall, while fig. 10 shows no sign of it. Examination of the organelle stained with iron haematoxylin shows that the typical spherical end-apparatus of a type 2 cell, with its clearly demarcated boundaries, has changed to the elongate form, with its distinct outer wall but ill-defined inner limits, which merge into the general cytoplasm of the cell.

The remainder of the cytoplasm is clearly divisible into two regions: the basal region is markedly fibrillar, even after fixation in a non-precipitant fixative such as Altmann's fluid. The fibres, which appear to be attached to the base of the cell, reach up to the level of the nucleus (fig. 12); they can be coloured with iron haematoxylin, eosin, and other dyes. In the middle region of the cell, between the nucleus and the end-apparatus, the cytoplasm is not fibrillar and is but weakly coloured by any dye. Iron haematoxylin and most basic dyes scarcely colour it, but toluidine blue does so weakly (fig. 10).

The orientation of the fibrils parallel to the main growth axis of the cell could be taken as an indication of a rapid intake of fluid by the cell from the haemolymph. The weak coloration by dyes suggests that the cytoplasm of the

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FIGS. 2–11

FIG. 2. Early type 2 cells. *Formaldehyde|dichromate: sudan black|carmalum.* The lipoid contents of the end-apparatus and of the cytoplasm appear black. The disorientation of the cells at this level is evident.

FIG. 3. Type 2 cells. *Alkaline phosphatase technique: eosin.* Alkaline phosphatase in the end-apparatus appears black.

FIG. 4. Type 2 cells. *Dichromate|osmium|acetic: iron haematoxylin.* The characteristically large end-apparatus and indented nucleus are visible.

FIG. 5. Type 2 cells. *Acid haematein technique.* Phospholipine in the cytoplasm, in the canalicules of the end-apparatus, and lining the walls of the tubule, appears black. The outer wall of the end-apparatus appears colourless.

FIG. 6. Type 2 cell.

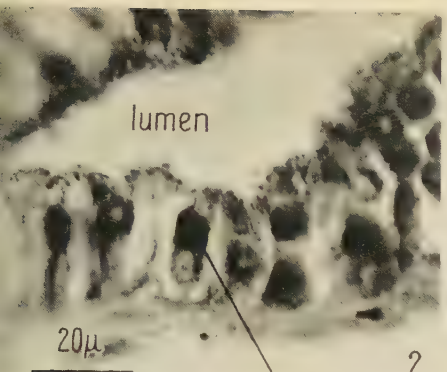
FIGS. 7 and 8. Transitional type 2/3 cells. *Methenamine|silver: toluidine blue.* The outer wall of the end-apparatus appears black; around this the striated body of the end-apparatus is faintly visible.

FIG. 9. Transitional type 2/3 cells.

FIG. 10. Type 3 cells. *Methenamine|silver: toluidine blue.* The outer wall of the end-apparatus appears black. The body of the end-apparatus is no longer present. In figure 10 a region below the end-apparatus which is coloured by toluidine blue appears grey.

FIG. 11. Type 3 cells. *Formaldehyde|dichromate: acid fuchsin.* The secretion within the end-apparatus appears as a cast of the end-apparatus shown in figure 10. There are no radial canalicules.

The scale on fig. 3 also applies to figs. 4–11.



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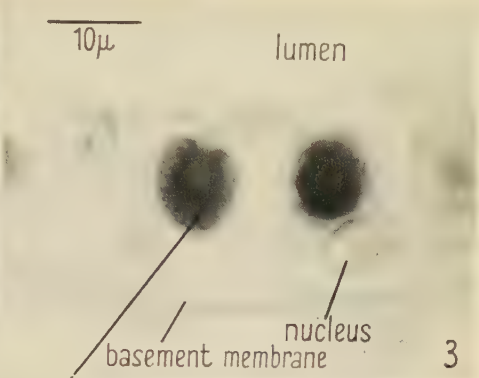

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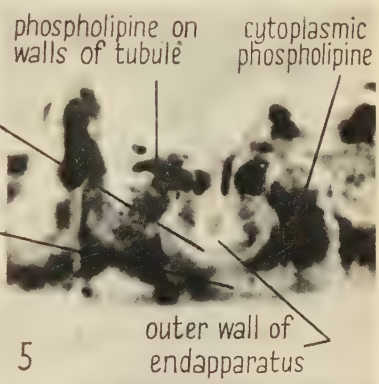

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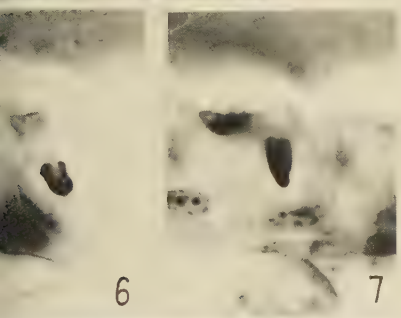

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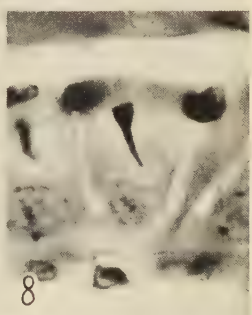

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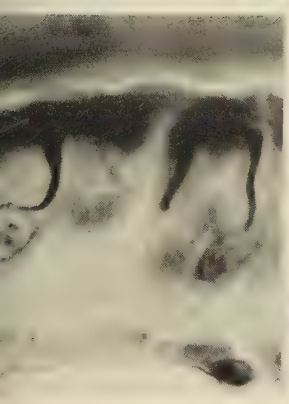

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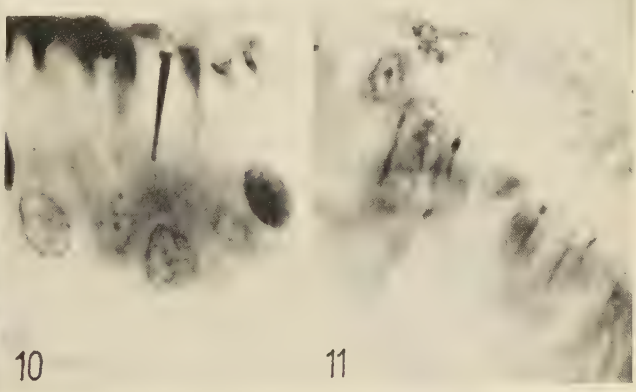

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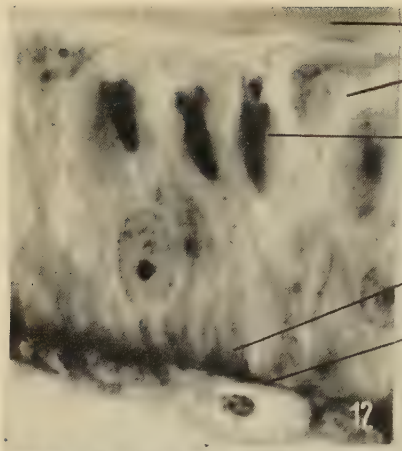
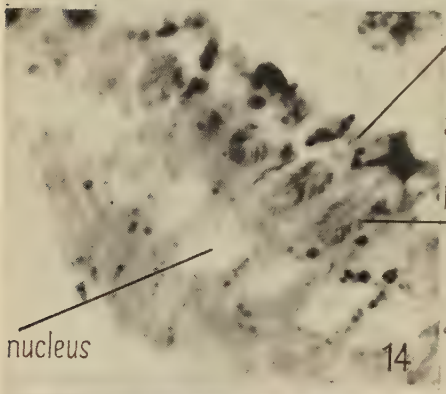
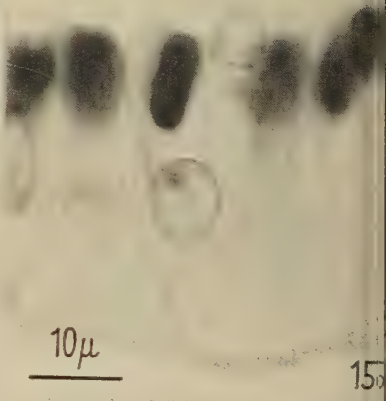
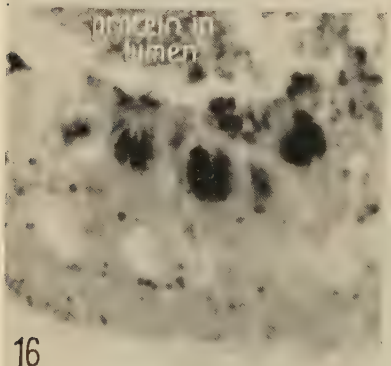

lumen
nucleus of chitinogenic cell
end-apparatus radial structure
inner wall of end-apparatus
basal fibrillation
basement membrane
12


15


protein leaving end-apparatus
phospho-lipine in end-apparatus
nucleus
14


10μ
15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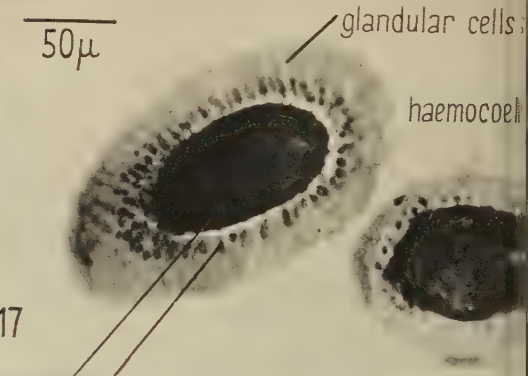
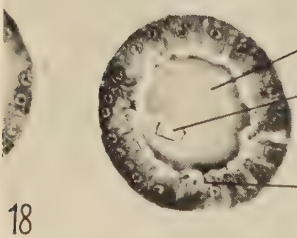
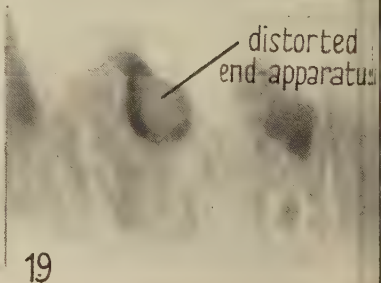

50μ
glandular cells
haemocoel
17


structural protein in lumen
calcium oxalate
end-apparatus
18


distorted end-apparatus
19

middle region is highly hydrated, certainly more so than the cytoplasm of type 2 cells, and the proneness of the middle region of the cells to collapse during fixation in (hypertonic) solutions is further evidence.

Phospholipines are present, but in far smaller quantities than in type 2 cells. There is a faint general positive reaction, showing that there is diffuse phospholipine in the cytoplasm. Particulate bodies occur among the basal fibres in the form of granules and rodlets of diameter not exceeding 0·5 μ; in the remainder of the cytoplasm they are present as spherical bodies ranging from 0·5 μ in diameter at the tips of the fibres up to 3 μ in diameter around the end-apparatus. The radially disposed phospholipine, so clearly seen in type 2 end-apparatus (fig. 5), is absent.

Baker's sudan black technique shows that lipochondria are disposed especially at the poles of the cell, where they occur in the form of granules or bipartite spheres of diameter ranging from 0·5 μ to 1·2 μ. In the middle region, similar organelles are present but in smaller numbers. Mitochondria are located among the basal fibres and around the end-apparatus.

There is an intense alkaline phosphatase reaction at the apical end of the cell, and this alone shows that some radial system remains within the reduced end-apparatus.

Type 3 cells clearly produce far less secretion product than either types 2 or 4, the regions that secrete structural protein. Such secretion as is produced is sparse and of a more granular nature than the other secretions of the gland. The cells themselves are twice as tall as the majority of the protein-secreting cells, and, while it is evident that these latter cells store no unfinished secretion within their walls, but rather store the finished secretion within the lumen, which is always full, the fact that the lumen in the region occupied by type 3 cells is more often than not empty supports the view that the increase in height of these cells occurs so that their secretion product may be stored

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FIGS. 12–19

FIG. 12. Type 3 cells. *Alkaline phosphatase technique: eosin.* The alkaline phosphatase in the end-apparatus is coloured black.

FIG. 13. Early type 4 cells. *Dichromate osmium/acetic: iron haematoxylin.* Overexposed negative to show radial structure of the end-apparatus and its well-defined inner wall.

FIG. 14. Early type 4 cells. *Acid haematein technique.* The ovoid end-apparatus, containing phospholipine which appears black, is visible.

FIG. 15. Early type 4 cells. *Alkaline phosphatase technique: eosin.* Alkaline phosphatase in the end-apparatus appears black.

FIG. 16. Later type 4 cells. *Acid haematein technique.* In this later stage the end-apparatus is becoming rounder than in figure 14; the radial canalicules remain.

FIG. 17. Early (left) and later (right) type 4 cells. *Formaldehyde dichromate: acid fuchsin.* General view of tubules of the gland. Protein, visible as threads in the end-apparatus, can be seen issuing into the lumen.

FIG. 18. Late type 4 cells. *Bouin: haematoxylin/eosin.* The dark appearance of the cells is due to cytoplasmic basiphilia. The end-apparatus is small. A crystal of calcium oxalate can be seen among the protein.

FIG. 19. Late type 4 cells. *Alkaline phosphatase technique: eosin.* The weak alkaline phosphatase reaction of the end-apparatus contrasts strongly with that of earlier figures.

The scale on fig. 15 also applies to figs. 12–16 and 19; that on fig. 17 also applies to fig. 18.

within the cell and released when required. Type 3 cells resemble in shape the cells of the right gland where storage of the secretion within the cell is easily demonstrable; they too are tall cells and the lumen of the gland is but sparsely filled. The end-apparatus is long and tubular and without the well-defined radial disposition of canalicules.

The presence of mitochondria and lipochondria in the base of the cell on the one hand, and around the end-apparatus on the other, is evidence that some synthetic activity is occurring there. The middle region is far less well equipped with these organelles, and it is there that the contents of the cytoplasm possess the power to react with the Nadi reagent after azide treatment.

The canalicules of the end-apparatus appear to be concerned with protein synthesis. The fact that they are reduced in the type 3 cells is additive evidence that this is the region responsible for secreting the oxidase. Where protein is formed (in the regions of the type 2 and type 4 cells), the secretion passes out of the cell through canalicules of the end-apparatus, but in this region alone this is not the case; fig. 11 shows the elaborated secretion, coloured with acid fuchsin, within the end-apparatus in the form of a cone such as would fit into the end-apparatus shown in fig. 10, and the absence of any secretion paths radially disposed about this cone is clear. The situation is quite unlike that shown in figs. 5 or 14.

The region occupied by the type 3 cells is smaller than the regions occupied by either type 2 or type 4 cells.

Type 4 cells. These cells constitute the bulk of the gland. Where they lie adjacent to type 3 cells they are tall, but there is a gradual decrease in height along the tubule. By far the majority are squat cells of dimensions similar to type 2 cells.

The most noticeable change that occurs is the reappearance of the thick-walled end-apparatus; this occurs while the cells are still tall. The difference between thin-walled end-apparatus of type 3 cells (fig. 12) is markedly different from the thick-walled end-apparatus of early type 4 cells (fig. 13). There is a well-marked inner edge, separating end-apparatus from the rest of the cytoplasm, and striations in the wall of the apparatus indicate that canalicules are present. This change in the end-apparatus occurs within the space occupied by a few cells.

That the secretion product of the cells passes out through canalicules in these cells, exactly as was the case in type 2 cells, can be seen in fig. 17, in which the protein has been coloured with acid fuchsin, and in fig. 14 the presence of phospholipines in the regions of the canalicules is clearly evident. Still retaining the radial striations, the end-apparatus changes from being ovoid to being more or less spherical in shape (cf. figs. 14, 16). Eventually the body of the end-apparatus decreases in volume and persists as a faintly acidophil U-shaped depression in the cell (fig. 18).

As the cells lose height the basal fibrillation is lost. Basiphilia increases, and the short cells are extremely basiphil. Experiments which would demon-

rate the presence of ribonucleic acid have not been made but that the asiphilia is due to its presence is inferred for a number of reasons. It is not significant that, while all histochemical tests for proteins show that the rotein in the lumen is far richer in amino-acid residues than the cytoplasm the cells, the test for arginine proves to be the exception. Arginine is known constitute a considerable fraction of the protein moiety of nucleoproteins, nd, indeed, Serra (1946) devised his modification of Sakaguchi's *in vitro* test r arginine, with the view to using this test so as to be able to infer from the sults of its use the presence of nucleoproteins. The fact that the cytoplasm a cell secreting an acidophil protein is strongly basiphil is, in itself, an indi- tion that basiphilia is due to the presence of proteins other than precursors the secreted protein, and suggests that the acidic nature of the protein (in e known absence of mucoproteins) is due to the presence of nucleoproteins. nderson's (1950) work on *Popillia* adds some weight to the theory, as does e fact that the cytoplasm of the cells is strongly coloured by pyronine.

Phospholipines steadily decrease in quantity as the cells decrease in height. he tall cells have phospholipines distributed in the cytoplasm in much the me way as in the type 3 cells, but the general diffuse reaction is weaker nd there is far more in the end-apparatus; there are fewer large granules in e cytoplasm and it is evident that these are often of a composite nature, the nospholipine surrounding a chromophobe droplet. In the short definitive cell ere is no diffuse phospholipine, and it is restricted to minute granules and dlets in the cytoplasm which appear to represent incompletely coloured itochondria. One or two large granules between 1 μ and 2·5 μ in diameter e present in each cell near the end-apparatus. The end-apparatus persists staining with acid haematein in the short cells, but the coloration is no ore intense than the coloration of the protein secretion itself in the control ctions, the lipoids from which have been removed with hot pyridine. All at can be said is that there is either no, or only very little, phospholipine the end-apparatus at this stage—there is definitely less than in earlier ages.

While phospholipines decrease in quantity, lipochondria and diffuse lipoid crease, and there is more lipoid in all type 4 cells than there is in type 3. oth in and around the end-apparatus diffuse lipoid is present, and in the tall lls there is diffuse lipoid in the basal region, below the nucleus. As the cell ortens, so these two sudanophil areas come together, leaving no uncoloured iddle region; the whole cytoplasm then contains diffuse lipoid. Lipochondria e fairly generally distributed: in the tall cells the diameter varies from 0·5 μ 1·5 μ, but in the short cells the maximum diameter becomes as large as 6 μ. itochondria are distributed in a similar pattern. At first they are concen- ted apically and basally, and later at random throughout the cytoplasm.

Alkaline phosphatase activity is again confined to the end-apparatus. At st the reaction is intense (fig. 15), but it falls off as the cell ceases to be nctional (fig. 19).

Discussion

The present interpretation of the anatomy of the left gland is not in serio disagreement with that of any previous author; it rather tends to confirm wh has been said, but also shows how very much more complex the organ is tha has been previously supposed. No previous author, for instance, has do more than briefly describe early and late stages of type 4 protein secretion.

The type of cell comprising the gland is not unusual in insects, and suc cells were already excellently figured by Leydig in 1859 and described occurring in dermal, anal, venom, salivary, and accessory sexual glands. Th the ductules of the female accessory sexual gland were lined with an 'inn wall' (chitinous intima) was known to von Siebold (1837) whose statement th the 'vesicular mass' (the cells) regularly showed a radiate texture extending the inner wall shows that he was even dimly aware of the presence of t ductules (longer in many cases than those in the colleterial glands) leading fro the end-apparatus to the lumen of the gland.

The presence of the chitinous intima throughout the gland indicates i ectodermal origin, and this is confirmed by the developmental studies of N (1929) and Quadri (1940). The female sexual accessory glands general possess such a structure and are of ectodermal origin: Metcalfe (1932) repor these conditions in Coleoptera, Cappe de Baillon (1920) in Orthoptera, an Bordas (1895) in Hymenoptera, but there is no homology with the larger the male accessory sexual glands (the utriculi of the cockroach (Ito, 1924 which, like that of Coleoptera (Anderson, 1950) and Hymenoptera (Bisho 1920) is mesodermal in origin, has no chitinous intima, and in which secr tion occurs as the result of imperceptible dissolution of the apex of the ce rather than through an end-apparatus. The conglobate gland of the ma cockroach, however, is of ectodermal origin (Ito, 1924).

Dufour (1841) observed that the 'substance cornéo-coriacée' of the blatt ootheca was derived from the colleterial gland (glande sérifique), but believe that the spermatheca (glande sébifique) served to provide a 'varnish' for i The cellular nature of the gland in *Blatta orientalis* was first figured b Duchamp (1878), but his diagram is rudimentary. He described the darke ing process of the ootheca and likened it to the change in colour of the cutic of a freshly moulted insect. He observed that the ootheca consisted of crysta bound together with a brown substance that he believed to be chitin. Kad (1879) described in detail the physical side of ootheca production, and notice that the left and right halves of the colleterial gland were unlike one anothe

Bordas (1909) made the first detailed study of the colleterial gland (glande arborescentes). He noted the unequal proportions of the left and right gland and described them and their secretion products. While noting that the le gland showed great differences in structure at different stages in the secreto process, he did not qualify this observation with clear descriptions of th stages. Evidently he did not discover the cells of the posterior part of th gland and, as his figures show, believed that cells resembling either type 3 c

arly type 4 cells were presecretory and cells resembling type 4 cells active or ostsecretory. Although he described the end-apparatus as occurring in the ght gland, he does not mention its occurrence in the left gland, a remarkable ict since he was aware of Leydig's work, and because his figures (especially g. 17) clearly show a dense apical region of the cell which undoubtedly epresents the end-apparatus. This omission may have been due to his opinion iat the secretory process in the left gland was analogous with that occurring i the intestine, and that the chitinogenic cells broke up, forming the secretion roduct, and were replaced by the, albeit rare, 'peritoneal' cells lying beyond ie basement membrane. His claim to have found nuclear debris in the lumen f the gland as a result of the breaking up of the chitinogenic cells can be iown to be erroneous by the use of Feulgen's technique. Although he was ware that the 'épithélium externe' (the glandular epithelium) decreased in ze as secretion proceeded, he does not pause to consider the significance of iis. There can be little doubt but that his interpretation of the nature of the ecretory process was erroneous. His observation that the crystals, which he escribed as being of calcium carbonate, found in the lumen of the left gland, ere formed there and not within the cells is correct. He described how these rystals were passed out of the gland and served to give bulk to the ootheca; ney were bound together by the mucilaginous secretion of the same gland.

Ito's (1924) description of the left gland was brief and dogmatic. His nalysis of the tubules into muscular coat, basement membrane, epithelium onsisting of two types of cells—glandular epithelial and chitinogenic and itinous intima is accurate, but his description of the component cells is rief: there is no figure of the left gland of *Blatta*, but he figures what are vidently type 4 cells as occurring in *Loboptera*. The statement that the glandu- r cells are cylindrical or cubical indicates that he had seen some of the various ypes of cell but did not localize them. Although he but briefly mentioned the nd-apparatus of Blattids, his figure of the end-apparatus in the mantid ccessory gland shows a clear understanding of their anatomy. He states that lobules of secretion, passing through the vesicles (end-apparatus) take on the spect of striae; and several of his figures representing their condition in iantids closely resemble my photomicrographs of *Periplaneta*.

Voy (1949) described the glands under the name 'appareil sébifique'. His gures and descriptions show clearly that those which apply to the gland hich he terms 'glande droite' (tubes du premier type) in fact apply to the ft gland of all other authors, and vice versa. To prevent confusion I use the ccepted term to denote the appropriate morphological entity. Voy's histo- gical details are sparse, but his figures of late and early type 4 cells are clear nd correct. There is no mention of type 2 cells, and I find that the posterior egion of the gland of *Blatta orientalis* which he describes differs from my bservations on *Periplaneta* (Brunet, 1951), but this may be a generic dif- rence. Pryor's work on the function of the glands was unknown to him, and e does not discuss their function.

Crystals of calcium oxalate occur, mixed with the protein, in the lumen of

the left gland in such large quantities as would suggest that they play some active part in either the formation of, or the function of, the ootheca.

Kadyi (1879) first analysed these crystals, which had been recorded by Duchamp in 1878, and Hallez (1909) confirmed the fact that they were calcium oxalate. Neither postulated the reason for their presence. Bordas (1908 and 1909) made the error of believing them to be crystals of calcium carbonate and attributed a structural function to them; as concrete consists of an inert filler, gravel, and a binding medium, cement, so the ootheca consisted of calcium carbonate crystals and a mucilaginous substance (the protein) respectively. There is probably some truth in this explanation; on the other hand the structural function of the crystals may be entirely incidental, their presence being due in the first instance to more fundamental reasons. Crystals of calcium oxalate are indeed used protectively in the plant kingdom to deter predation by molluscs; the presence of these crystals interferes with the smooth running of the radula. But the ootheca is brittle and would almost certainly be shattered by a preying animal, in which case the crystals could have little deterrent effect against this occurrence; and the insolubility of the crystals and their being enclosed in an insoluble tanned protein matrix could not lead to their causing unpalatability.

A saturated solution of calcium oxalate would have a pH value on the alkaline side of neutrality, and herein lies the most likely explanation of the function of the oxalate. Ewins (1910) found that the Comessatti test for adrenalin (a diphenolic substance) worked best in alkaline solution: adrenalin can be oxidized to a red compound by mercuric chloride, and Commessatti when he discovered this had used tap-water as solvent. Ewins showed that this reaction also occurred in the presence of many salts of strong bases with weak acids. Ewins pointed out that this was in agreement with the work of Euler and Bolin (1909) who had shown that several so-called laccases, promoting oxidation of phenolic compounds (e.g. the laccase of *Sedum*) were not in fact enzymes at all but calcium salts of mesoxalic, citric, and malic acids: and from this he concluded that the rapid oxidation of phenolic compounds is dependent on the presence of free hydroxyl ions in solution. Calcium oxalate is probably present with the protein so that when the diphenolic substances from the right gland diffuse into it the pH is at an optimum for enzymatic and/or spontaneous oxidation of the phenol. Ito (1924) was of the same opinion, but his conclusion, in the absence of any reason for arriving at it, was evidently speculative.

Oxalates are known to occur within the Malpighian tubules of insects and it has not been disproved that the presence of oxalate in the left gland is due to its acting as an auxiliary excretory organ.

The glandular cells of the colleterial glands share in common with integumental dermal glands the possession of an end-apparatus. In the vestibulum surrounding the outlets of the colleterial glands is an organ composed of massed dermal glands of the integumental type (Brunet, 1951) and at first it seemed likely that there might be a material connexion between these and

the secretory cells of the colleterial glands, but this is definitely not so: both left and right glands have a region of non-secretory cells which separate the dermal gland organ from the secretory cells. Integumental dermal glands have, in general, a conspicuous duct leading from the end-apparatus, sometimes reaching 200 μ in length, and round the duct lie an unknown number of cells supposed to have secreted the duct. In the colleterial glands, where the end-apparatus is present, the duct is usually vestigial, as, for example, in type 2 cells of the left gland (it is, however, 7 μ long in type 2 cells of the right gland), but this marked difference is bridged by many examples of intermediates in other similar glands of ectodermal origin (Beier, 1933). The glandular epithelial cells of the colleterial gland are evidently cells related to dermal gland cells, which have undergone similar morphogenetic processes, and are accompanied by but one other type of cell (the chitinogenic) which probably represents the unmodified epidermal (hypodermal) cell, and not the cells found on the duct of integumental dermal gland cells.

Exactly what function the end-apparatus performs is not completely clear. It is undoubtedly a complicated structure in the cells which secrete structural protein; it is no mere filter, for the properties of substances which have passed through it into the lumen differ from those present in the cytoplasm; this fact and the fact that alkaline phosphatase is concentrated there show that the last stages of metabolism occur in the end-apparatus. The anatomy of the end-apparatus in structural protein-secreting regions suggests that it may have yet another function: the minute radial canalicules in it, through which the secretion has to pass, as well as providing a large surface for enzyme action, could act as spinnerets, the shearing forces acting on the micelles passing through them and causing partial physical denaturation, as is known to occur in secreted silk (Meyer, 1950; Ramsden, 1938). The absence of canalicules in regions which do not secrete structural protein adds some support to this hypothesis. In these regions, the oxidase-secreting region of the left gland, and in the cells of the right gland, histochemical tests and staining reactions show little or no difference between the secretion when it is in the cytoplasm and when it is in the lumen, which suggests that less active metabolism occurs in the end-apparatus of these regions than occurs in the region secreting structural protein.

The occurrence of alkaline phosphatase in the body of the end-apparatus is consistent with previous reports of its presence at secretory surfaces (Bradfield, 1951); and though the present results are not detailed enough to confirm Bradfield's statement that the presence of alkaline phosphatase and ribonucleic acid tend to be mutually exclusive in the cytoplasm, they are not in disagreement: the main protein-secreting region, type 4 cells, has a strongly basiphil cytoplasm, indicative of the presence of ribonucleic acid, while the end-apparatus is acidophil and contains all the cytoplasmic phosphatase. There is apparently no correlation between the presence of alkaline phosphatase and phospholipines.

REFERENCES

Anderson, J. M., 1950. Biol. Bull., **99,** 49.
Baker, J. R., 1933. *Cytological Technique,* 1st ed. London (Methuen).
—— 1945. Ibid., 2nd edn. London (Methuen).
—— 1946. Quart. J. micr. Sci., **87,** 441.
—— 1947. Ibid., **88,** 115.
—— 1949. Ibid., **90,** 293.
—— 1950. Proc. Linn. Soc., London, **162,** 67.
—— 1951. Personal communication.
Bauer, H., 1933. Jahrb. Morph. mikr. Anat., **33,** 143.
Beier, M., 1933. *Orthopteroidea,* in Schulze, P., *Biologie der Tiere Deutschlands.* Berlin.
Bishop, G. H., 1920. J. exp. Zool., **31,** 225.
Bordas, L., 1895. Ann. Sci. nat. Zool., **19,** 1.
—— 1908. C. R. Acad. Sci., **147,** 1495.
—— 1909. Ann. Sci. nat. Zool., **9,** 71.
Bradfield, J. R. G., 1951. Quart. J. micr. Sci., **92,** 87.
Brunet, P. C. J., 1951. Ibid., **92,** 113.
Burtner, H. J., and Lillie, R. D., 1949. Stain Tech., **24,** 225.
Cain, A. J., 1947. Quart. J. micr. Sci., **88,** 151.
—— 1948. Ibid., **89,** 229.
Cappe de Baillon, P., 1920. Cellule, **31,** 5.
Chèvremont, M., and Frederic, J., 1943. Arch. Biol., **54,** 590.
Danielli, J. F., 1946. J. exp. Biol., **22,** 110.
Duchamp, G., 1878. Rev. Sci. Nat., Montpellier, **7,** 423.
Dufour, L., 1841. Mem. Acad. Sci., Paris, **7,** 265.
Euler, H., and Bolin, I., 1909. Zeitschr. f. physiol. Chem., **61,** 1.
Ewins, A. J., 1910. J. Physiol., **40,** 317.
Giroud, A., and Bulliard, H., 1933. Protoplasma, **19,** 381.
Gomori, G., 1939. Proc. Soc. exp. Biol., **42,** 23.
—— 1946. Am. J. clin. Path., **16,** Tech. 117.
Hallez, P., 1909. C.R. Acad. Sci., **148,** 317.
Hirsch, G. C., 1948. Verhandl. d. Deut. Zool., Kiel, **7,** 226.
Holtfreter, J., 1946*a*. J. exp. Zool., **101,** 355.
—— 1946*b*. Ibid., **102,** 51.
Hotchkiss, R. D., 1948. Arch. Bioch., **16,** 131.
Ito, H., 1924. Arch. d'Anat. Microsc., **20,** 343.
Jackson, E. L., 1944. Organic Reactions, **2,** 341.
Jacobson, W., 1939. J. path. Bact., **49,** 1.
Kadyi, H., 1879. Zool. Anz., **2,** 632.
Keilin, D., 1936. Proc. Roy. Soc. B., **121,** 165.
Lillie, R. D., 1947. J. Lab. Clin. Med., **32,** 910.
—— 1950. Anat. Rec., **108,** 239.
Lison, L., 1931. Arch. Biol., **41,** 344.
—— 1936. *Histochimie animale.* Paris (Gauthier–Villars).
—— and Fautrez, J., 1939. Protoplasma, **33,** 116.
McManus, J. F. A., 1948. Stain Tech., **23,** 99.
Metcalfe, M. E., 1932. Quart. J. micr. Sci., **75,** 49.
Meyer, K. H., 1950. *Natural and synthetic high polymers,* 2nd edn. London (Interscience).
Nel, R. I., 1929. Quart. J. micr. Sci., **73,** 25.
Nicolet, B. H., and Shinn, L. A., 1939. J. Am. Chem. Soc., **61,** 1615.
Palade, G. E., and Claude, A., 1949. J. Morph., **85,** 35 and 71.
Pryor, M. G. M., 1940*a*. Proc. Roy. Soc. B, **128,** 378.
—— 1940*b*. Ibid., **128,** 393.
—— Russell, P. B., and Todd, A. R., 1946. Bioch. J., **40,** 627.
Quadri, M. A. H., 1940. Trans. R. Ent. Soc., London, **90,** 121.
Ramsden, W., 1938. Nature. **142,** 1120.
Ries, E., 1935. Zeit. Zellforsch. mikr. Anat., **22,** 523.
Romieu, M., 1925. C. R. Acad. Sci., **180,** 875.

ROSKIN, G. I., and STRUVE, M. E., 1947. Stain Tech., **22,** 83.
SERRA, J. A., 1946. Stain Tech., **21,** 5.
SIEBOLD, C. T. VON, 1837. Arch. Anat. Physiol., p. 381.
STACEY, M., 1946. Adv. carboh. Chem., **2,** 161.
THOMAS, O. L., 1948. Quart. J. micr. Sci., **89,** 333.
TRIM, A. R., 1941. Bioch. J., **35,** 1088.
VOLKONSKY, M., 1927. Bull. d'hist., **5,** 220.
VOY, A., 1949. Ann. des Sci. Nat. Zool., **11,** 269.

On the Nutrition of *Ochromonas*

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Summary

A species of the chrysophycean genus *Ochromonas* from England was grown in pure culture and compared with similar strains isolated in the United States. Although these organisms possess chromatophores with chlorophyll, they need organic substances not only as a source of nitrogen compounds, but as independent carbon and energy sources; they also require certain growth factors. Peptones are better as nitrogen sources than single amino-acids, few of which give satisfactory results. The utilization of inorganic nitrogen could not be demonstrated. As carbon sources sugars, alcohols, and fats, and, to a lesser degree, acetate, are suitable. Growth factors contained in liver extract, milk and yeast autolysate, and in smaller amounts in most peptones, are indispensable. Enzymes hydrolyzing starch, sucrose, fat, and protein are excreted.

Ochromonas ingests and digests starch grains, casein, oil droplets, and small organisms. Neither bacteria nor yeast nor small algae were found able to support growth of any of the strains, even in the light. An additional carbon compound and possibly special vitamins are required. The hypothesis is put forward that phagotrophy chiefly serves for obtaining chemical compounds which the cell cannot synthesize itself and which have the character of vitamins. Phagotrophy therefore supplements the other means of nutrition, in the main qualitatively, while sugar, &c., supplement it quantitatively and are indeed indispensable.

The same media which support ample growth in the light do so also in the dark. This shows that photosynthesis is of little importance for the growth of the organisms, although it keeps them alive for long periods, while in the dark they soon die.

Correlated with the manifold ways of acquiring food, there is also a remarkable variability in the size and shape of the cells, and still more in the dimensions and pigmentation of the chromatophores, and in the reserves stored as leucosin, volutin, and oil. These features which now prove to depend so much on conditions have previously been widely used in the diagnosis of species. The basis of classification should therefore be revised. Apart from the stigma, which is specific, use ought to be made only of those characters whose variation with conditions is reasonably well known.

The extraordinary nutritional versatility of *Ochromonas* is considered to be a primitive character. From ancestors with a mixed nutritional habit, phototrophic, phagotrophic, and saprotrophic flagellates seem to have evolved, each with only one of the three modes of nutrition that are combined in *Ochromonas*.

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Contents

Introduction

OWING to the lack of pure cultures very little is known of the physiology of the Chrysophyceae. *Synura* alone has been grown free from bacteria (Mainx, 1929), but only in soil extract, and has not been used for experiments on nutrition. The investigations of Hardin (1942) on *Oikomonas*, an apochlorotic member of the class, are therefore the only source of information available. While *Synura* is predominantly photosynthetic, *Oikomonas* lacks assimilatory pigments and requires organic nutrients; with peptone as a nitrogen source, both glucose and glycerol are utilized.

The first culture experiments with pigmented and colourless Chrysophyceae were made by Meyer (1897), who found that his organisms multiplied in sugar solutions. He had no pure cultures and does not seem to have made subcultures. In the same year Bohlin (1897) described an organism which he called *Chloramoeba* and believed to be a monadoid member of a group later named Heterokontae or Xanthophyceae; in this he was followed by all subsequent authors. It was probably an *Ochromonas* with greenish chromatophores, such as are found exceptionally in other Chrysophyceae (cf. Lewis, 1913; Gavaudin, 1931). These authors have described their forms as belonging to a xanthophycean genus *Chlorochromonas*. In the absence of cysts this ascription is only supported by the greenish colour, which is also recorded for Chrysophyceae (Fritsch, 1935, p. 508). That the organism was in fact an *Ochromonas* is supported by the presence of an inclusion drawn by Bohlin (p. 514, fig. 6*c*) as oil but probably leucosin, which has not been established in Xanthophyceae, and also by the nutritional behaviour of the organism. Bohlin was able to grow it on various organic substances in successive subcultures in the dark, where it became almost colourless. As he did not exclude bacteria, the compounds he used are not of great interest, but it is worth mentioning that a wide range

f carbohydrates, higher alcohols, glycosides, &c., were successfully employed. These important results are in conformity with those of Meyer and with the ater work of Doflein. (On green Chrysophyceae see also Böcher, 1945.)

Doflein (1922 *a*, *b*, 1923) proceeded in the same way as Meyer, and his xperiments are likewise only of value in that they show that organic sub-tances—sugar, or compounds originating from it, as a result of bacterial netabolism—favour the multiplication of these flagellates; they do not permit onclusions to be drawn regarding the compounds actually consumed. Doflein's notion that *Ochromonas* and its apochlorotic counterpart *Monas*, are sugar flagellates', in the same sense (Doflein, 1916) as *Polytomella* and *Poly-oma*, was unsound. The latter in fact utilize organic acids, mainly acetic acid produced in Doflein's experiments by other micro-organisms) and are unable o make use of sugar (Pringsheim, 1921, 1935; Pringsheim and Mainx, 1926); he same might possibly be true for the Chrysophyceae cultivated by Doflein.

The nutrition of such organisms, particularly of those possessing chromato-hores with chlorophyll which also ingest and digest particulate food, is of reat importance but cannot be elucidated without experiments based on the ehaviour of pure cultures. The question of the significance of phagotrophy oupled with phototrophy is one of particular interest; even in colourless lagellates the phagotrophic mode of nutrition has only as yet been investigated n one instance, that of *Peranema* (Chen, 1950). Although the possibility of earing flagellates with an animal-like nutrition in the absence of other organ-sms was predicted 30 years ago (Pringsheim, 1921, p. 135), it has only recently een accomplished. The methods developed in recent years have now been pplied to the study of *Ochromonas*.

Material and Methods

The investigations began in 1940 with a clone isolated from a Cambridge arden pond and determined as *Ochromonas variabilis* H. Meyer (1897), but ot obtained in pure culture. It was found to thrive in soil-water cultures with rganic matter, but did not multiply in inorganic media even when these were upplemented with soil extract. In the dark, the chromatophores became pale nd almost invisible.

This organism seems to be identical with the first brown chrysophycean to e isolated, bacteria-free, in an organic medium and subsequently used for he investigations on which this publication is based. It was found in material rom a red film on a stone from the mountain lake, Malham Tarn, in Yorkshire. By transferring single cells, after washing, to soil-water tubes, good growths vere obtained, from which Dr. T. Y. Chen made pure cultures in 1950 by vashing with the help of capillary pipettes. His medium contained 0·2 per ent. glucose and 0·1 per cent. Difco proteose peptone and permitted sub-ulturing in series, although growth was poor.

After Dr. Chen had returned to Pekin, a better medium was sought, and a umber of differently constituted mixtures were tested. Since *Ochromonas* loes not grow on agar, all cultures were made in liquid media and inoculated

with a loopful from the surface of a previous culture or, where uniform inoculation was essential, with drops after shaking. The cultures were grown in Pyrex test-tubes, except where the need for more abundant material made necessary the use of conical flasks. For most experiments, series of tubes were suspended on a rack, illuminated by fluorescent tubes arranged horizontally and parallel to each other in the vertical plane. At a distance of 30 cm. a test-tube filled with water had a temperature $\frac{1}{2}$–1° C. higher when illuminated than when the illumination was switched off. The light intensity, measured with a Weston 'Master' Exposure Meter with integrating attachment, was about 25 units. The illumination was continuous for 16 hours daily. For experiments at a constant temperature higher than that of the room, water-baths were used which were electrically heated, illuminated, and stirred.

At a later stage three more strains of unicellular chrysomonads became available. Two of these were isolated in pure culture by Dr. L. Provasoli (Haskins Laboratory, New York): one from a mixed culture of fresh-water algae, another from a crude culture supplied by Dr. H. P. Brown (University of Oklahama). The third was supplied as a culture with bacteria by Dr. R. A. Lewin (Osborn Botanical Laboratory, Yale University), who occasionally observed stalked goblets and identified his organism as the insufficiently described *Poteriochromonas stipitata* Scherffel. In posteriorly elongated cells like that shown in fig. 1 (p. 80), similar but very delicate structures can be demonstrated with dehydrating agents in all four strains; but there remain differences. In Scherffel's figures the protoplasts are shown to occupy a place at the opening of the goblet which also must have been much better defined. All four strains, compared in pure cultures, are very similar, not only morphologically but also in their nutrition, although there are differences in their particular vitamin requirements. There is at present no reason to separate these organisms from *Ochromonas* or to give them different names.

Nutrition

Vitamin requirements

In Chen's original medium, containing 0·2 per cent. glucose and 0·1 per cent. Difco proteose, multiplication was not abundant. The cultures were but faintly coloured and contained unhealthy-looking cells with small, pale chromatophores. Supplementation of Chen's medium with Difco beef extract, Difco yeast extract, or soil extract, and the use of other peptones, &c., proved inadequate as long as the cultures remained bacteria-free, though growth in infected cultures was sometimes excellent. Eventually a medium containing liver extract was found to give an immense improvement. After 2 days at room temperature the cultures in this medium were turbid, especially in the top layer. Multiplication was maintained, so that after some weeks a thick deposit of brown cells formed. Three brands of liver extract, one American (from E. Lilly and Co.) and two English (from Glaxo and from Oxo Laboratories), had the same effect. I am much indebted to the firms that helped me.

The great improvement in growth after the addition of liver extract indi-

cated that a substance or substances not present in sufficient amounts in proteose peptone was needed for healthy growth. By using a 0·2 per cent. glucose+0·2 per cent. tryptone basic medium in which no growth occurred, t was possible to determine both the lowest and the optimum concentrations of liver extract for the growth of *Ochromonas*. When Glaxo standardized liver extract, extracted with ether, was used, 0·2 per cent. seemed to be about optimum. Below this concentration the multiplication rate decreased continuously with decreasing concentration. Slight growth occurred at 0·01 per cent., but none was observed without liver extract. At concentrations higher than 0·2 per cent. multiplication was about the same up to 1 per cent.

The relatively high concentration of extract necessary for good growth shows that the amount of the relevant growth factor or factors present in the extract is low, or that the organism's requirements are considerable. The non-standardized liver extract from Oxo gave about the same quantitative results as the Glaxo extract. With liver extract autoclaved in alkaline solution and then neutralized, growth still occurred, though at a reduced rate. This suggests that more than one substance may be needed, one of which is affected by this treatment and may well be B_{12}.

From Difco yeast extract, unlike laboratory-made yeast autolysate, the substances important as growth factors appear to be absent. Yeast autolysate was made by incubating baker's yeast with toluene+chloroform at 37° C. for a few days and subsequently using the supernatant fluid. This was more effective when autoclaved than when Seitz-filtered, although a considerable precipitate formed during autoclaving.

The difference between yeast autolysates sterilized by filtering and those sterilized by autoclaving extends to the colour of the growths. Young cultures are in general pale; pigmentation develops gradually, not in parallel with increase in numbers, so that the darker colour is not due solely to the greater number of cells present. Pigmentation also depends in part on illumination: cultures grown in the dark tend to be only faintly coloured, though almost colourless cultures can be obtained in the light. Control of pigmentation seems chiefly to be exercised by the vitamin content of the medium. Unheated yeast autolysate supports growth, although the cultures do not become quite as dense as with liver extract, but they remain almost white. When, however, the yeast autolysate is autoclaved, growth, and particularly pigmentation, become much more pronounced: deep olive brown to sepia-coloured cultures are obtained. The same colour appears with high concentrations of liver extract, but of this deep shade only when yeast extract or unheated yeast autolysate is added. At least two factors seem therefore to affect pigmentation, and the effects of these factors on growth and on pigmentation do not proceed in parallel. In these respects again all four strains behaved in the same way. When after some time cultures become dark sepia or almost black in colour, this is due to the presence in the culture fluid of a water-soluble brown substance. Since it is also produced by pale growths in the dark, this is not likely to be phycochrysin, the ill-defined pigment from Chrysophyceae.

In test-tubes with liquid media, growth is first seen 2–3 mm. below th surface. This becomes still more apparent when the organisms are distribute in liquid agar, preferably of low agar content. A clear zone is then seen, under neath which there is a zone of optimum multiplication. *Ochromonas* also grow well in stab-cultures, but very poorly on agar-surfaces.

Carbon sources

Meyer (1897, pp. 46 et seq.) succeeded in cultivating a number of Chrysc monads in sugar solutions, among them four species of *Ochromonas*. His state ment that sugar was essential, no multiplication occurring without it, is o considerable interest. Doflein (1922*a*), experimenting in a similar way, wa inclined to assume direct assimilation of sugar, although he held it desirabl to examine the possibility of its conversion into other compounds befor utilization. Since he lacked pure cultures this question could not be decided

Chen's first pure culture contained glucose. When this was replaced b acetate, growth was much reduced, so that *Ochromonas*, unlike *Polytomella* may well be called a sugar-flagellate (Doflein, 1916) and does not belong t the acetate-flagellates (Pringsheim, 1935).

Apart from glucose, other carbohydrates, for example sucrose and starch were equally suitable: both are hydrolysed in pure cultures with the formatio of reducing sugars. On starch-agar plates, translucent areas developed aroun clumps of cells transferred to them, and by pouring dilute iodine solution o the agar, colourless halos with reddish margins on an ink-coloured groun were made visible. Amylase is therefore excreted. Fructose and galactose wer also satisfactorily utilized; but lactose and xylose were ineffective.

In order to determine the most favourable concentration of glucose, a serie of tubes containing graduated percentages of glucose were prepared. At relatively high concentration of liver extract, for example, 0·2 per cent. an still more at 0·5 per cent., with no peptone, some growth occurred even with out the addition of sugar, due no doubt to carbohydrates from liver. Thi growth was not appreciably increased by adding 0·012–0·025 per cent. glucos and was only significantly improved by adding 0·05–0·2 per cent. When th concentration of liver extract was reduced to 0·1 per cent., and nitrogen defi ciency prevented by adding 0·2 per cent. Bacto tryptone, only slight multi plication took place without sugar; 0·012 per cent. glucose had a definite effec in promoting growth and multiplication was almost optimum at 0·025 per cent and most prolific between 0·05 and 0·2 per cent. glucose. When the sugar con centration was further increased, a reduction in the growth-rate ensued: 0· per cent. glucose was the highest concentration for luxuriant growth; 0·3–0· per cent. was slightly less favourable; and at 0·6, 0·8, and 1·0 per cent. th multiplication rate dropped more and more, although even at 4–5 per cent appreciable multiplication was still observed. This became very slight at 6 an 7 per cent. and ceased at 8 per cent. Glucose therefore supports growth a concentrations between 0·01 and 6 per cent.

Of the alcohols, 0·2 per cent. glycerol, mannitol, and ethanol were tested

of them can replace glucose, although multiplication was never quite as ofuse as in glucose. In order to determine their effects more exactly, the nount of liver extract was further reduced and with it the influence of carbo- drates already present in the preparation. With 0·2 per cent. Bacto peptone d 0·01 per cent. Oxoid liver extract, some degree of multiplication was still served with 0·025 per cent. glycerol, but not without it. 0·1 per cent. and still tter 0·2 per cent. of this alcohol improved growth considerably; this dropped higher concentrations, more than 1 per cent. being definitely harmful.

Lactate, tartrate, malate, and citrate at various concentrations had no effect, d acetate supported growth only moderately well.

More successful than the cultures with organic salts were those with fat, hich proved an excellent source of carbon and energy. A stable emulsion of per cent. olive oil and 3 per cent. egg lecithin in distilled water was used: is could be autoclaved without visible alteration. The oil droplets are so nall that a medium containing 1 per cent. of this emulsion, that is, 0·15 per nt. oil, is not turbid but has the appearance of very dilute milk with a arked Tyndall effect: it appears bluish against a dark background and llowish against the light. Usually 1–2 drops of this emulsion were added eptically to 10 c.c. of sterile medium in a test-tube.

In a solution of 0·1 per cent. tryptone and 0·1 per cent. Oxoid liver extract pplemented with 1 per cent. oil emulsion, all the *Ochromonas* strains multi- ied well, and a brownish coloration appeared, beginning near the surface of e fluid. All three substances are necessary for the organisms to grow. Seitz- tered yeast autolysate could be used instead of liver extract, but pigmenta- on was much reduced in the absence of the liver factor. After 5 or 6 days at om temperature, or 4 days at 21–30° C., the medium cleared, the Tyndall fect disappeared, and the organisms accumulated on the bottom. The same edia also supported growth in the dark. Oil can therefore replace sugar but oes not contain the growth factors essential for multiplication.

Growth with oil was at least as abundant as with the same percentage of ucose and could be further improved by the addition of liver extract with ast autolysate. Since multiplication was vigorous, and since the organism ed only in the upper regions of the test tube, it seemed unlikely that the sult could be due merely to the *ingestion* of oil droplets by the cells; there ust presumably be an effect at a distance.

This assumption was tested by means of an agar medium, which prevented th oil and organisms from moving. Agar with peat extract at pH 5·5 was ade turbid with oil emulsion and poured into sterile Petri dishes. It was then ssible to observe clarification of the agar around an inoculum of *Ochromonas* lls, which in this medium multiplied only slightly. By using an agar-oil ulsion mixture with tryptone and liver extract inoculated in a liquid state 40° C. and immediately cooled with cold water, numerous small colonies uld be obtained. These developed mainly in a zone between 3 and 6 mm. om the surface, particularly in the dark, while they reached deeper down in e light, although considerably diminished in size. Growth was better when

an almost fluid agar of only 0·2–0·3 per cent. was used. The turbidity gradually disappeared, beginning with the superficial regions. These experiments show that a lipolytic enzyme is excreted and penetrates through the agar gel.

The oil-digesting capacity of *Ochromonas* is worth further investigation. It may provide a valuable means of supplying nutrients that are insoluble in water.

Nitrogen sources

Without a special source of carbon, such as those already mentioned, no multiplication took place with any of the peptones tested, not even when these were supplemented with beef extract, yeast extract, or yeast autolysate, solutions of some of which gave good growth in the presence of minute amounts of sugar. In peptones, *Ochromonas* showed definite multiplication only after the addition of relatively large concentrations of liver extract (which of course contains glycogen).

In the presence of sugar various nitrogen sources had different effects. Some growth occurred with Difco casitone, Difco Bacto peptone, Difco proteose peptone, silk peptone Roche, Evans peptone, Oxoid bacteriological and mycological peptone, but other preparations, such as Difco tryptone and Difco protone, did not support growth. All of these, with the exception of protone, were excellent (at a concentration as low as 0·2 per cent.) when supplemented with Oxoid liver extract or with similar preparations. Those peptones which permit some growth without liver extract probably contain small quantities of the growth factors supplied by the latter. With blood serum and purified milk casein, good growth was likewise only obtained in the presence of liver extract; the media, which were very turbid and lumpy after sterilization, gradually became quite clear. On agar and gelatin, *Ochromonas* does not grow, but after transferring a quantity of cells to the surface of milk agar or casein agar, clear zones formed around the inoculum and in gelatin holes appeared, showing that proteolytic enzymes are excreted.

Amino-acids again are very different in their effects. In the presence of 0·2 per cent. glucose and 0·1 per cent. Oxoid liver extract, 0·1 per cent. glycin, asparagin, and alanin proved moderately good sources of nitrogen, while leucin and cystin had no effect. Glycin could also be used with acetate. Without liver extract, no amino-acid produced growth in a medium containing only sugar and dilute Benecke's solution.

Inorganic nitrogen as nitrate and ammonium salts did not improve growth in a sugar liver extract medium.

Hydrogen-ion concentration

Chen's medium containing glucose and Bacto proteose was markedly acid and so was the improved medium with glucose, Bacto tryptone, and liver extract. Since multiplication was abundant, neutralization was evidently unnecessary. Moreover, it was found that no growth occurred after adjusting the pH to 7·0 with sodium carbonate. In order to determine the pH range o

growth, a basic medium with 0·1 per cent. glucose, 0·1 per cent. tryptone, and 0·1 per cent. Oxoid liver extract was used. It is an excellent medium, although distinctly acid (pH about 4·0). In adjusting such media to a particular H-ion concentration it should not be forgotten, as Schoenborn (1950) seems to have done, that after autoclaving and cooling to room temperature the pH is regularly found to have shifted, mostly so that the pH values of a series come to be closer together, the extremes being much reduced. Our solution was adjusted with sodium carbonate to pH 8·0, 7·5, 7·0, 6·0, 5·5; after autoclaving the values were 6·8, 6·4, 5·8, 5·5, 5·3.

The upper limit for growth of one strain was at about pH 6, that is, still in the acid region; no growth took place at pH 6·4. The optimum appears to be near pH 5·5. A lower limit was not reached. In a second series growth was only very slight at pH 3·6 but perceptible at pH 4·1. These results were tested several times in various series of experiments with the same and other strains; they were confirmed each time. Growth of *Ochromonas* in this medium is therefore possible between pH 4 and 6. *Ochromonas malhamensis* is thus a distinctly, though not extremely, acidophilic organism.

Reserve substances

In most of the cultures, the cells were full of reserve substances: leucosin, oil, volutin, and granules of unknown nature. Droplets which, partly because of their small size, could not be distinguished from oil, were found to stain with neutral red and methylene blue. Their colour varied: in the former stain either red or orange; in the latter, blue or mauve. Spherical, highly refractive droplets, and sometimes larger drops, were shown by means of sudan III, sudan black, and osmium tetroxide, to be oil, containing unsaturated fat. In older cultures, these drops disappeared, presumably being used up in the course of metabolism. They were found in all well-fed cultures in media with sugar or glycerol but were more numerous and larger in cultures with oil emulsion (fig. 1). When multiplication was rapid, reserves did not accumulate and, some time after the oil-containing medium had become clear, very little oil was left in the cells. If, however, the multiplication rate was low owing to vitamin deficiency (for instance in cultures with unheated yeast autolysate and oil emulsion), the quantity of oil in the cells became considerable, so that the whole cell-body was crammed with droplets, some of them flowing together to form relatively large drops (fig. 2).

I did not succeed in distinguishing oil taken in with food from that which had been digested and redeposited, as was done in *Peranema* (Chen, 1950) with the help of stained oil as food. The colour of droplets small enough to be digested did not show sufficiently after staining with sudan black. There is, however, no doubt that deposition takes place, since cells on a fat-free diet also contain oil.

A much larger body, not regularly spherical, lying in the posterior part of the cell, often almost filling the cell completely, was identified as leucosin. The chemical nature of this substance, so characteristic of the Chrysophyceae,

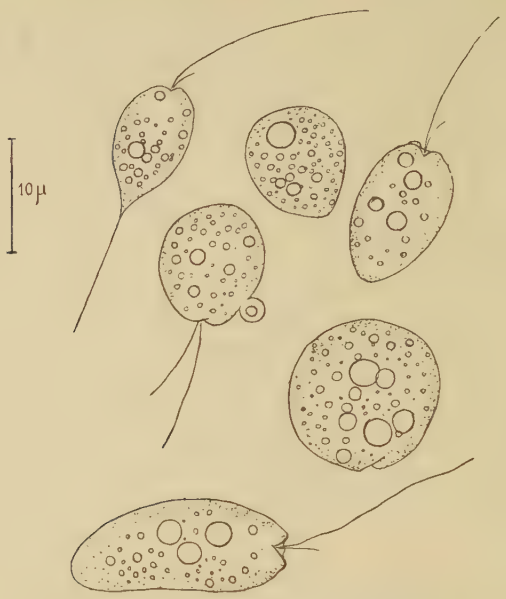

Fig. 1. *Ochromonas malhamensis*, containing large quantities of oil after cultivation in Bacto tryptone 0·1 per cent.+liver extract 0·1 per cent. −oil emulsion. One cell has just formed a vesicle surrounding an oil drop; another cell is attached to the substratum by a delicate cytoplasmic strand.

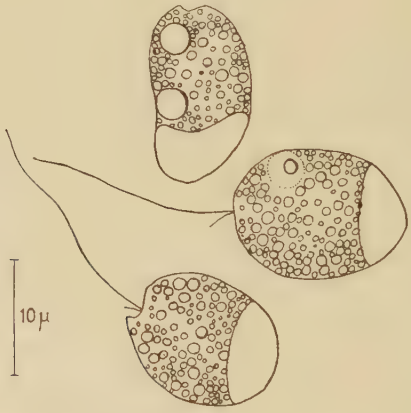

Fig. 2. *Ochromonas malhamensis* containing an enormous number of oil droplets and leucosin. The organism was grown in 0·1 per cent. Bacto tryptone+oil emulsion, so that multiplication was restricted by lack of vitamins. This was the appearance after a fortnight. In one cell, two very large oil drops are visible; in another, a food vacuole with an oil droplet.

s not known. It does not give any chemical reaction useful for its identification. Its physical properties are also unknown except its appearance under the nicroscope. This conveys the impression of a syrup in a vacuole lacking space ufficient for it to assume, by surface tension, a completely spherical shape. This interpretation as a vacuole is supported by the observation that it occaionally stains orange with neutral red (cf. Chadefaud, 1947). When the cells re killed, and when reagents such as ethyl alcohol, acids, alkalis, mercuric hloride, or other fixatives are applied, the leucosin bodies disappear, although t was observed that when they were large the place they had occupied appeared as a vacuole with aqueous contents after fixation with osmium tetroxide.

Leucosin is a substance formed in vast amounts by planktonic organisms in akes and in the sea, and it would therefore be of much interest to know somehing of its chemical nature. Since the time of Klebs (1883), leucosin has enerally been regarded as a polysaccharide. Its appearance does in fact reemble that of glycogen as found, for example, in yeast, except for the absence f staining with iodine. Korshikoff's (1929) assumption that the substance is f a protein nature is not supported by any observation or reasoning. Unfortuately the name 'leucosin' has also been given to an albumen occurring in ereals, and the last syllable of the name suggests that it is indeed a protein. t would therefore be well to change the name. The substance from *Chilomonas* nvestigated by Hutchens and others (1948; cf. Hutner and Provasoli, 1951, . 47) and named leucosin, was starch; the members of the Cryptomonadineae enerally produce this.

A correlation between the quantity of leucosin in the cells and the presence f sugar in the medium (fig. 3, A, B) (Meyer, 1897; Doflein, 1923) cannot in self be regarded as sufficient evidence of the polysaccharide nature of leucoin, so long as cells grown for comparison in a sugar-free medium have not een obtained. A diminution of the quantity of this substance in old cultures hows only that it is a reserve, gradually used up when the nutrient solution ecomes exhausted. In cultures with acetate, the amount of leucosin is lower han in those with sugar, but so is the growth rate. Glycerol gives better rowth and more leucosin, particularly when multiplication comes to an end, ut never as much as in the presence of carbohydrates. In cultures with oil nstead of sugar in the medium, leucosin is stored in addition to oil, and its uantity can be considerable (cf. fig. 2). When glucose, even as little as 0·02 er cent., is added to a culture growing for instance on glycerol, the quantity f leucosin is increased within a day.

In testing *Ochromonas* for a possible content of Fehling reducing compounds, with or without hydrolysis, sugar-cultures were not at first expected o be suitable: the delicacy of the organisms seemed to forbid thorough washng in the centrifuge. After good cultures with glycerol had been achieved, an ttempt was made to detect the presence of reducing sugars. The best medium or this purpose was found to be: glycerol 0·2 per cent.; Difco casitone 0·2 per ent.; Oxoid liver extract 0·1 per cent. If not too sparsely inseminated, a oo c.c. conical flask with 250 c.c. of the medium developed, in about 10 days

at room temperature and in light of moderate intensity, a thriving cultur with a thick olive brown sediment of cells and cell detritus (largely leucosin which could be used for the test. A neutralized hot-water extract gave m Fehling reaction. Attempts to hydrolyse the substance were made in variou ways: by heating the originally weakly acid solution at various temperature and for various times, and repeating this after the addition of differen

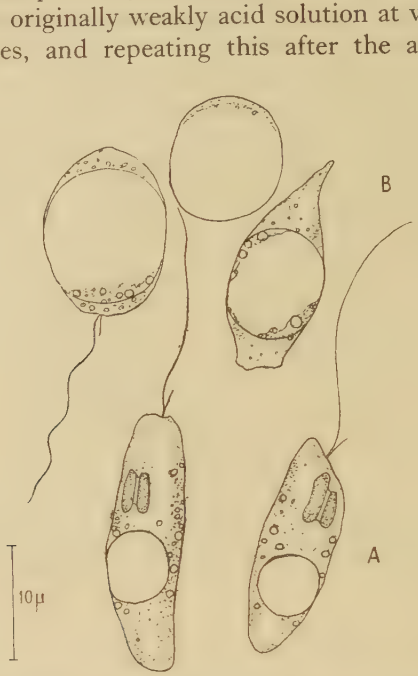

FIG. 3. *Ochromonas malhamensis*: A, glucose 0·1 per cent. — Bacto tryptone 0·1 per cent. — Oxoid liver extract 0·1 per cent.; B, glucose 2 per cent. + Bacto tryptone 0·2 per cent. + Oxoi liver extract 0·05 per cent. In A, more actively swimming individuals than in B, although no all of them are motile. The quantity of leucosin in B is much larger than in A; the chromato phores are much reduced and in most of the cells invisible. Note in A the suggestion of longi tudinal rows of oil droplets.

amounts of hydrochloric acid; by autolysis, keeping a quantity with toluen and chloroform at room temperature or 35° C. for one or several days; by th action of amylase, proceeding in a similar way but adding taka diastase. I these two latter instances, the Fehling test resulted in a marked turbidity bu not the characteristic yellow-red precipitate. The existence of reducing sugars directly or after hydrolysis, could not therefore be established in this way.

At this stage a communication by von Stosch (1951) came to my knowledge He demonstrated in *Synura* (Chrysophyceae) and various diatoms, where re serves similar to leucosin are known to occur, the precipitation of crystallin masses resembling inulin, under the influence of dehydrating substances, par ticularly diacetin (glycerol diacetate). Repeated attempts to get the same resul with *Ochromonas* rich in leucosin failed. Neither concentrated sea-water, no sodium chloride, glycerol, or glucose gave by dehydration the effect describe by von Stosch. Only by mixing culture material with the same volume o

diacetin, and warming at 65° C. for 20 to 30 minutes, could spherulites be obtained. This and other observations seem to make von Stosch's dehydration hypothesis doubtful, and to indicate some concomitant chemical process.

Endeavours to obtain a Fehling reducing sugar were now renewed. The above-mentioned glycerol medium was supplemented with glucose to produce a cell-material rich in leucosin. The medium was: 0·2 per cent. glycerol, 0·1 per cent. glucose, 0·2 per cent. Difco casitone, and 0·1 per cent. Oxoid liver extract. After heavy inoculation, considerable growth was obtained in 3 days when the cells contained much more leucosin than in previous attempts. Centrifuging and washing with glass-distilled water proved much less harmful than was expected. Most of the cells remained in a healthy state and moved about normally. The sediment was dark olive-brown. The supernatant fluid gave only a feeble Fehling reaction. By warming in a water-bath at 65° C. for 90 minutes the cells were killed, changed colour to greenish olive, and clumped together, making filtration through paper possible, while a great percentage of living cells pass through. The filtrate gave a strong Fehling reaction, and so did water with which the residue on the filter had been washed. The experiment was twice repeated with new cultures, and tests were added in which a short boiling was used instead of heating to 65° C. In the filtrate no reducing substances were then present, while even 25 minutes at 65° C. sufficed to hydrolyse the substance in the cells, presumably leucosin.

These results indicate that killing of the cells by applying a temperature which does not destroy the enzyme system, as boiling does, sets the enzyme free to hydrolyse leucosin with the formation of reducing sugars. Now, since (1) leucosin production is enhanced by the presence of sugar, (2) leucosin is transformable into a crystallized substance similar to inulin, (3) reducing substances (sugar?) can be obtained by an enzyme contained in the cell, the polysaccharide nature of leucosin becomes very probable. I suggest that this substance should be called *chrysose*, and that this term should include the probably identical substance obtainable from diatoms, which are taxonomically related to the Chrysophyceae.

Particulate food

Food vacuoles are regularly found in a percentage of the cells of *Ochromonas*. In nature, their contents vary and can only be recognized shortly after organized particles have been ingested. Algal cells, almost as big as *Ochromonas* itself, may be included among the contents, and small bacteria, fungal conidia, and the like are also to be found. In experiments, cells of *Chlorella vulgaris* and *Saccharomyces exiguus* were found suitable for watching the actual capture of food particles. If these are too small or actively motile, observation is difficult. If they are too big or too long, the phenomena are not so characteristic. Quickly swimming *Ochromonas* individuals do not seem to ingest food (cf. for *Anthophysa*, Pringsheim, 1946*a*, p. 322), although the process may simply have been overlooked; but since the flagellum plays an important role in the capture of particles, it is quite likely to be inefficient for this purpose when used in

swimming. Sessile or slowly moving *Ochromonas* cells are seen to cause small particles approaching them to spin. When the particles approach the insertion of the flagella they often rotate at great speed (cf. Gavaudin, 1931, p. 291, for *Chlorochromonas*). They may escape the vortex generated by the long flagellum, but usually they suddenly stop moving and in the next second are seen to lie within a clear vesicle formed like a blister at the surface of the cell-body. This vesicle is probably open at first, so as to let the particle in. Gavaudin (1931, p. 291) describes and figures a protoplasmic cylinder which closes over the particle and becomes a vesicle surrounding it. I was not able to observe this structure in *Ochromonas*. The vesicle is soon withdrawn into the cytoplasm. The place where ingestion takes place in *Ochromonas* is always near the anterior end of the cell, where the protoplasm tends to form small outgrowths or short pseudopodia.

The formation of the vesicle (cf. fig. 1) takes place so rapidly that it can hardly be followed by the eye. Dark-ground illumination is not very helpful, though it makes flagellum, surface layer, and vesicle more easily visible. The vesicles are from the beginning much larger than the particle engulfed and contain a clear fluid which surrounds it. They can be observed as food vacuoles inside the body until only small refractive residues of the prey are left. A cell can take in several particles, one after the other, whatever the state of nutrition may be. Thus cells from an old culture, where scarcely any ingestible particles remain, and which seem to live wholly autotrophically, will ingest food offered to them in the same way as those from a culture with an abundance of food particles.

Very long objects, such as *Ankistrodesmus* cells or bacterial rods or chains, are partially ingested, the vesicle seeming to be pierced by such objects, although it is scarcely possible to establish whether a thin layer of cytoplasm surrounds them or not (fig. 4). Often there are a number of particles within one food vacuole. This is very often so when small bacteria are ingested.

In order to establish what kinds of particles are ingested, *Ochromonas* was fed with various objects. Water-colour paints were suitable, because of the smallness of their particles. Carmine, indigo, Indian ink, vermilion, cobalt blue, sepia, and cadmium yellow were all taken in, apparently indifferently. Sand grains, calcium sulphate, calcium carbonate, and powdered glass were not taken (compare Chen's results with *Peranema*, 1950). The difference apparently lies in the weight of the particles. Those which the twirling flagellum cannot throw against the soft surface at the anterior end of the cell are not incorporated.

In mounted preparations with ingestible particles, the number of food vacuoles present seemed to be much smaller on the following day than after a short period of feeding.

Digestion was difficult to observe, since removal of cells into a hanging drop without particles (Chen, 1950) was not feasible because of the smallness of the cells. Bacterial cells, and those of small algae and yeasts, were used for these observations, and *Ochromonas* was also fed with well-defined particulate sub-

stances: rice starch, coagulated casein, and oil-emulsion (cf. p. 77). Little could be seen beyond the fact that all these were readily ingested. Starch and oil drops were taken with about the same speed as yeast and *Chlorella* cells. After some hours they did not appear to have changed. The next morning very few such particles were present in food vacuoles, and such vacuoles as were found might have been formed shortly before examination. The same was observed with casein and fat.

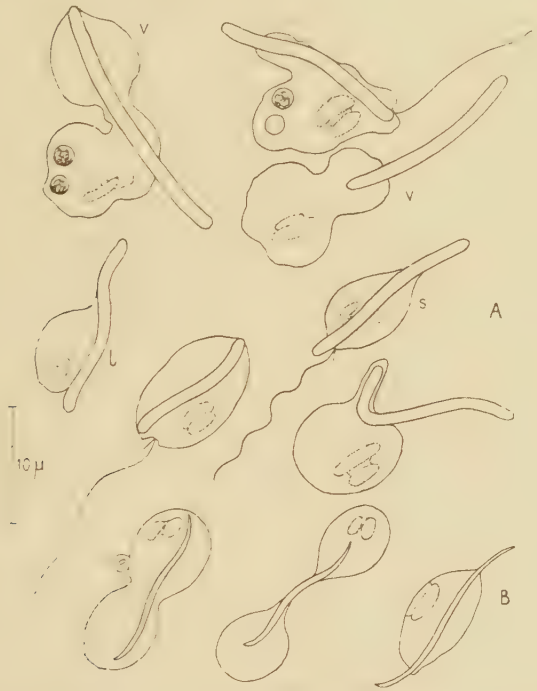

Fig. 4. *Ochromonas malhamensis*: the flagellates are feeding on relatively long organisms: in A, on *Lineola longa* Pringsh., a large bacterium; in B, on *Ankistrodesmus angustus* Chodat et Oettli, a small green alga. (*v*) transparent ingesting vesicles; in (*l*) the bacterium became detached after a few seconds; (*s*) actively swimming away with the bacterium inside.

Attempts to determine the pH of food vacuoles were no more successful. Cells of *Saccharomyces exiguus* were stained with indicators by heating in aqueous solutions of neutral red, methyl orange, Congo red, and phenolphthalein. No change towards an acid reaction was observed in the vacuoles, and no means were found of detecting a change to the alkaline side, because no suitable indicator stained the yeast cells sufficiently for this purpose. While in *Paramecium* Congo-red yeast becomes blue (Pringsheim, 1928), no such change was observed in *Ochromonas*, nor in *Peranema* (Chen, 1950), so that the two flagellates so far investigated behave alike in this respect but differently from ciliates and amoebae (Mast, 1942; Mast and Bowen, 1944). The food vacuoles contain much liquid and are in this respect different from those of

Peranema (Chen, 1950), where food particles are immediately in contact with the cytoplasm.

In pure cultures of *Ochromonas*, without any other organisms or solid particles present, a certain number of specimens regularly showed food vacuoles containing remnants of bodies which can only have been weak *Ochromonas* eaten by the more vigorous individuals. The same phenomenon is found in pure cultures of *Peranema* (Chen, 1950), and also in *Glaucoma piriforme* and *G. vorax*. If in pure culture one of the above-mentioned organisms is provided with bacteria or other suitable food, the number of food vacuoles formed increases enormously within a few minutes.

Temperature and illumination

Ochromonas grows well at room temperature, and also at lower temperatures down to about 15° C. If the temperature is further reduced, growth gradually becomes slower and slower, so that a minimum limit cannot be given. An increase in temperature, on the other hand, speeds up growth, up to 30° C. A further rise in temperature to 32° C. brings the organism very near to the maximum temperature for multiplication: growth is almost nil. Since it is more convenient to keep cultures at room temperature than in a lighted water-bath, cultures at other than room temperatures were only used for special studies.

The temperature characteristics of growth are the same for this organism in the dark as in the light. *Ochromonas* grows well without photosynthesis if suitable media are employed. In the dark, glucose gives much better results than glycerol, while in the light the difference is less pronounced. Particularly at 30° C., the development of cultures on glucose may not at first be much slower in a dark incubator than in a lighted water-bath. Any medium which supports growth in the light also permits multiplication in the dark. A retarding influence due to the exclusion of light is, however, always observed sooner or later. Whether this is only due to lack of substances produced in photosynthesis or also to lack of oxygen has not been determined.

An appreciable difference is found in the colour of the cultures: those in the dark are much less pigmented and usually almost colourless or only yellowish —not brown, as in the light. Another pronounced difference between otherwise identical cultures in the dark and in the light is the longevity and persistent high density of the latter, while with the exclusion of light growth soon comes to an end, though a percentage of the cells remain alive for weeks.

Ochromonas is not noticeably sensitive to differences in light intensity and grows in dim light almost as well as near fluorescent tubes. It is not readily damaged by strong natural light, but is content with the little the cultures receive in front of a north window in the English winter, provided that the temperature is not too low.

Comparison with the nutrition of Hardin's Oikomonas

When we endeavour to compare the nutrition of *Ochromonas* with that of other organisms, Hardin's results stand closest and are the most important.

His pioneer work on pure cultures of *Oikomonas* (1942) reveals striking similarities to, and some differences from, the results with *Ochromonas*. Unfortunately the method of obtaining his organism which he describes as easy failed to give a result. It is not altogether clear whether Hardin's material was in fact an *Oikomonas*, since he does not state how he identified it. Hardin's figures (1942, p. 468) show *Monas*-like flagellates in various states. He was not familiar with such organisms, as is shown by the sentence (p. 468): 'The hyaline body in the posterior part is probably a reserve body'—that is, leucosin seems not to have been known to him.

Hardin used a medium with proteose and glycerol but found (p. 471) that proteose with glucose was slightly better. This was the precedent followed by Chen, and their results are in agreement. As for sugars, Hardin found glucose better than sucrose; both were better than glycerol—results which again agree with mine. His results with N-compounds are, so far as they go, also the same as mine. Bacto neo-peptone was best; then followed proteose, tryptone, yeast extract, Bacto peptone, and amino acids, in descending order of suitability. In a few respects, e.g. in the matter of optimum pH, there are differences. For *Oikomonas*, the reaction has to be neutral or only slightly acid, while none of the *Ochromonas* strains grew in neutral media.

In a later paper Hardin (1944, p. 196), following Butterfield, put forward an accumulation theory to explain the observation that his *Oikomonas* multiplied abundantly in pure culture in media of higher concentration, for example, in 1 per cent. glycerol with 0·5 per cent. proteose, though when this medium was diluted five times, growth was only good if suitable bacteria were present. The bacteria are supposed to 'act as accumulators or concentrators of food'. Since similar results can be obtained with non-phagotrophic organisms, such as *Astasia ocellata*, *Euglena gracilis*, *Chlorogonium elongatum*, *Polytomella caeca*, &c., another explanation has to be looked for. This may be found in the production by the bacteria of growth factors of which not enough is present in the dilute media. In the experiments with *Ochromonas*, for example, proteose permitted only poor growth, but this could be improved considerably by the presence of certain bacteria, and also by liver extract and yeast autolysate.

Hardin also found a considerable stimulation of growth in *Oikomonas* by the addition of small quantities of agar. This was not found in *Ochromonas*, where agar cannot replace liver extract.

Morphology

In consulting the descriptions of species of *Ochromonas* given by Meyer (1897), Pascher and Lemmermann (1913), and Doflein (1922*a*, 1923), or the identification table prepared by Huber-Pestalozzi (1941), in order to determine our forms, none were found to be satisfactory. Almost all the characteristics made use of by the various observers change much, in one and the same clone, according to conditions. Size and shape of cells, form and pigmentation of chromatophores, nature of cell surface, length of flagellum

compared with body length, and still more the amount and kind of reserve substances—all undergo far too much modification for them to be used for identification as attempted by these authors. Cysts which might have been useful were never formed by any of the four strains in numerous cultures, not even in soil-water media, which provide more natural conditions than the others and yield cultures which remain active for a very long period.

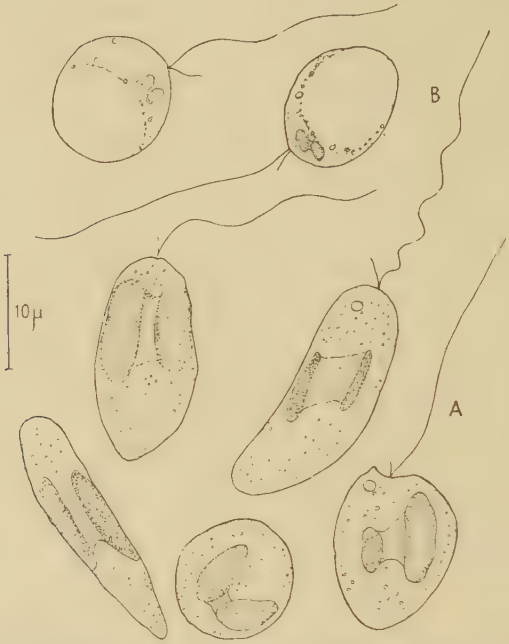

Fig. 5. *Ochromonas malhamensis*: A. showing chromatophore well developed after a fortnight's growth in the following medium: glucose 0·15 per cent. + beef extract 0·08 per cent. + proteose 0·1 per cent. + Oxoid liver extract 0·02 per cent. Note also flagella and contractile vacuole. B. chromatophore reduced in size and colour; large leucosin body, as observed after 3 weeks in the dark in the following medium: glucose 0·1 per cent. + proteose 0·1 per cent. + Difco yeast extract 0·1 per cent. + Oxoid liver extract 0·025 per cent.

The form first isolated and mainly used has, like the others, no eye-spot and only one chromatophore. Such a species has not been described. The name *Ochromonas malhamensis* is therefore proposed, derived from the place where it was found. The species is near to *O. variabilis* Meyer (1897), which, however, is supposed to possess two chromatophores. It is not impossible that Meyer's observation was at fault. The chromatophore of *O. malhamensis* is composed of two longitudinally arranged elongated lobes which, under dry objectives, may simulate two separate chromatophores but under oil immersion are seen to be connected by a bridge (fig. 5A). Only in large specimens, evidently preparing to divide, and in abnormal individuals, have the cells more than one chromatophore.

Meyer and Doflein have observed the chromatophores of *Ochromonas*

species to be pale and small when there was plenty of organic food (fig. 5B), but brown and considerably larger in mineral solutions, in which, however, the organisms did not multiply. The same is true of *O. malhamensis*. Young cultures, during quick multiplication in suitable organic media, appear yellowish in colour with cells containing small, faintly coloured chromatophores. The characteristic olive-brown hue develops later, after the main period of multiplication is over; no doubt, the concentration of organic nutrients is then reduced. For this deepening of colour to take place, light is required.

In the dark, cultures remain lighter than those of like density grown in the light and are never really brown. When the culture fluid appears brown, this is due to the death of many cells with ensuing diffusion of pigment into the medium; a white layer of cell residues accumulates on the bottom, leaving the supernatant fluid clear and therefore appearing darker when undisturbed than after stirring.

In the light also, coloration of the growth depends on the composition of the medium. With sterile filtered yeast autolysate, growth may be quite dense, but the cells produce very little pigment, so that in bulk the growth appears colourless, and chromatophores are invisible in most of the cells. Several dozens of such seemingly apoplastidic cells were isolated. The majority developed into brown populations; the rest of the cultures remained sterile. No colourless race was obtained.

The appearance of the cells varies, often in one and the same clone-culture, while cells in old cultures do not necessarily differ, even after weeks of growth, from those in younger ones. The majority of the cells are usually rounded (ovoid or spherical) in shape, often with a depression in front where the two unequal flagella emerge. Actively swimming cells are not as common in this species as in others. Such are more slender, often spindle- or club-shaped, tapering or pointed posteriorly and containing less reserve substances. Cells which move with the help of their flagella are present in nearly every population, but most of the cells are no longer than they are broad and show little tendency to move, the flagella being used mainly for bringing food particles towards the front of the cell.

Such rounded cells, the greater part of the volume of which may be taken up by leucosin (fig. 6), gradually accumulate as a slimy, brown sediment in the culture. They remain alive for many months, although the percentage of dead, decomposing individuals increases with time. After transfer to a new medium, the reserves are consumed, cell division is resumed, and soon a great number of free-swimming individuals are seen, forming swarms near the surface. The more or less spherical non-motile cells have a diameter of about 6–8μ, while the elongate motile cells are on the average 12μ long and 4μ wide.

The two flagella are of unequal length, one being one-and-a-half times to twice the body-length, the other one less than a quarter as long as the body. They are not so readily observed as in other chrysomonads but show well under dark ground illumination. The longer one rotates funnel-wise and is

not generally curved in swimming as in related organisms. The short flagellum is difficult to see when active.

Near the front end of the cell is a relatively large contractile vacuole; it seems to be absent under certain conditions. The position of the nucleus varies considerably. The cytoplasm of the anterior part of the cell seems to be more fluid than the rest and is able to form pseudopodia of constantly varying shape; these emerge and are withdrawn incessantly. It is rare to see

FIG. 6. *Ochromonas malhamensis* with much leucosin and little oil; chromatophores reduced, invisible in most cells; occasional food vacuoles; as seen after 3 days' growth in the following medium: glucose 0·2 per cent.+Bacto tryptone 0·1 per cent.+yeast autolysate (half concentration, unheated, sterilized by filtration).

the back end drawn out into a fine thread, though many similar organisms fasten themselves to solid bodies with a posterior cytoplasmic strand (see fig. 1). Amoeboid movement occurs only after irritation (fig. 7).

Leucosin, which has been discussed as a reserve (p. 79), has to be mentioned again here as a morphological feature. While oil is usually scattered in the cytoplasm without obvious regularity (though occasionally the droplets tend to be arranged in a longitudinal row (fig. 3A)), leucosin always occurs as a single body. This, when not too large, is situated in the posterior part of the cell, not only in our organisms but generally in Chrysophyceae; and even when it fills almost the whole volume of the cell, the remaining cytoplasm is collected in the region of the cell where the flagella are inserted. In elongate cells the leucosin body may be almost cylindrical. The singleness and the posi-

ion of the leucosin body indicate that it has a certain morphological signi-
cance. In a marine chrysomonad called *Pavlova gyrans* by its discoverer,
R. W. Butcher, the cells, resembling a slender *Ochromonas*, regularly contain
wo leucosin bodies. Two per cell are also indicated by Chadefaud (1947) for
is filamentous chrysophycean, *Nematochrysopsis*. These observations appear
o show that the leucosin body is not just a vacuole situated anywhere in the
ell, filled with a leucosin solution: it has a definite morphological substratum.

Fig. 7. *Ochromonas malhamensis*: cells of irregular shape, strongly amoeboid. This form seldom found and is only temporary; it occurs in mounted preparations and may possibly e caused by pressure of the coverslip. Medium: glucose 0·1 per cent.+beef extract 0·1 per ent.+yeast autolysate. Above: division stage.

Discussion

The predominant impression obtained from this general study is one of xtraordinary nutritional versatility exhibited under varying conditions. This rganism is able to use almost every way of acquiring food found in any micro-rganism. Organic particles of every description are quickly taken up and igested in food vacuoles, so that protein, starch, and fat are utilized. The me substances are also hydrolysed outside the body, no doubt by enzymes ffusing into the medium. Disaccharides are hydrolysed and assimilated. In ddition to carbohydrates, acetate, alcohols, and amino-acids are also utilized its metabolism. This means that phagotrophic and saprotrophic nutrition e both well developed in this organism. They permit *Ochromonas* to dispense ith radiant energy in the visible range, so that, when well supplied with rganic nutrients, it develops almost as well in the dark as in the light. It is ot to be concluded from this, however, that the chromatophore with its con-nt of chlorophyll does not fulfil any function under natural conditions. This comes clear when one compares old pure cultures kept in the light with ose kept in the dark. While the latter die out in a few weeks with signs of

starvation, those in the light live for many months, continuously, thoug slowly, multiplying, with some of the cells in active movement; but most c the cells are stationary, using their flagella for catching food-particles derive from dead cells. They develop well-pigmented chromatophores, presumabl carrying out photosynthesis. Yet without organic substances in the medium there was no multiplication in any of the mineral solutions tested, not eve when these were supplemented with soil extract, yeast extract, or beef extract

It appears, then, that photosynthesis helps to keep *Ochromonas* alive ove long periods in pure culture, though it is evidently not sufficient to provide al the organic compounds required for multiplication. The observed need fo special growth factors bears out the inadequacy of photosynthesis.

The mode of nutrition is not holozoic: that is, *Ochromonas* does not liv entirely on ingested organisms. Feeding with small algae, yeast, or bacteria di not (in my experiments) suffice to give cultures, either in the dark or in th light. *Chlorella pyrenoidosa*, *Saccharomyces exiguus*, and *Aerobacter aerogene* were the organisms used for most of the experiments; when these were adde to a mineral solution or soil extract with mineral salts, *Ochromonas* multiplie very little at best. When, however, a medium containing sugar and peptone but lacking the necessary growth factors, was inoculated with the organism mentioned, and these were allowed to multiply before *Ochromonas* was added the latter grew well. This shows that certain growth factors, produced by th growth of these organisms, are able to support the growth of *Ochromonas*. Fo the kind of mixed nutrition exhibited by *Ochromonas* there is no term: photo sapro-phagotrophy describes it, though it sounds inconveniently clumsy.

The fact that growth-promoting substances are known to be present i yeast and bacteria is in favour of the notion that phagocytosis in *Ochromona* is essential in furnishing, in addition to ordinary food requirements, com pounds which the *Ochromonas* cell is unable to synthesize.

Ochromonas secretes a number of enzymes for the hydrolysis of sucrose starch, protein, and fat, not only into food vacuoles but also into the medium It would appear, therefore, abundantly equipped for the utilization of al kinds of food. The question arises whether this versatility is a primitive featur or one acquired more recently by gradual adaptation.

Although the common possession of remarkably similar biochemica mechanisms and nuclear structures is in favour of the origin of all organism with the cell type of organization from a common ancestor, no such original perfectly primitive creature is known. There are at least three main relatively lowly groups: the Myxophyceae (to which the Myxobacteria and Spirochaete may be related (see Pringsheim, 1949)), the Bacteria, and the flagellates, be tween which there are no transitional forms. Only the flagellates have de veloped into morphologically complicated higher organisms: Algae, Fungi Cormophyta, and Metazoa; they themselves, in spite of common features, fal into five divisions: Chrysophyceae+a few heterokontal forms, Euglenineae Chloromonadineae, Cryptomonadineae+Dinomonadineae, and Volvocales Which of these groups has preserved truly primitive characters and whic

ontains members most similar to those from which plants and animals proper ave evolved? There can be no doubt that the Chlorophyceae are related to ne Volvocales. These, however, are too far advanced in their entire organiza- on to be near to the ancestors of other organisms. All the other flagellates, ith the exception of certain Chrysomonadineae, are still more specialized, owever, so that a further development cannot have originated from them. his leaves only the Chrysomonadineae, themselves comprising an abundance f variously adapted forms. They include, on the one hand, pigmented, algal rganisms, on the other, many colourless forms, some of them definitely nimal-like. Probably also derived from chrysophycean ancestors are the leterokontae (for the most part true algae) and the Diatoms.

The cytoplasm of the Chrysomonadineae is translucent and fluid, and its urface soft with a kind of mutability of contour different from the metaboly f Euglenineae, Chloromonadineae, and some ciliates, but also to be found in olourless flagellates and rhizopods. This variability in shape and non-rigid urface are necessary, though not sufficient, for the intake of solid particles, hich is often restricted to certain areas of the protoplast. Since the colourless agellates are derived from pigmented ones by the loss of their chromatophores Pringsheim, 1942, 1948), the most primitive members of the Chrysomonadi- eae should have the following properties: one chromatophore; living singly hat is, not forming organized colonies); cell-body naked and able to engulf ood particles; one long and one short flagellum like the majority of the species. uch an organism would be referred to the genus *Ochromonas*. This is the norphological aspect of the matter.

'Primitive' and 'derived' organisms both have essentially the same basic iochemical organization. It is nowadays obvious that chemo-autotrophic rganisms, for instance, need not be, and generally are not, primitive. They ave the same biochemical make-up, and often the same morphological ap- earance, as certain phototrophic or saprotrophic forms, to which they are vidently related. Biochemically, they cannot possibly be envisaged as primi- ive. On the contrary, the ability to utilize the oxidation energy of inorganic ompounds is an acquisition over and above the 'ordinary' metabolism of the ell. This is borne out by the ability of the majority (perhaps of all) of the hemo-autotrophic organisms to live on organic compounds. Similarly, strict hoto-autotrophy perhaps does not exist. A plant which synthesizes proteins, tarch, fats, nucleoproteins, chlorophyll, and other complicated compounds, uilding them up from the primary products of photosynthesis, cannot be hysiologically more primitive than a saprotrophic organism. One should ather ask the question: why are substances, formed and utilized in internal netabolism, not also used when supplied from without? Separation of an nner metabolism from substances supplied exogenously must be regarded as secondary acquisition.

If that is so, then photo-autotrophism would be expected in the higher, erived, and more complicated algae rather than in the lower, more primitive orms, which should be able not only to photosynthesize, but also to make use

of suitable preformed organic compounds. So far as is known, this is the case. Ambitrophism, that is, the ability to live either on preformed or on photosynthetically produced organic compounds, is found in the more primitive Chrysomonadineae, Xanthophyceae, Volvocales, Euglenineae, Chlorococcales and Ulotrichales, but nowhere in the morphologically more complicated members of these groups, and not in any member of the higher Algae, for example the Chaetophorales, Siphonales, Siphonocladiales, Charales, Rhodophyceae and Phaeophyceae.

The versatility of nutrition in a *Chlorella* would then be regarded as more primitive than the strict photo-autotrophy of *Cosmarium*, and the nutrition of *Ochromonas* as more primitive than the photo-autotrophy of *Mallomonas*. *Peranema*, with its entirely animal-like nutrition, would be an extreme case of derived adaptation from an ancestor of a generalized nutritional type. The great versatility of *Ochromonas* can therefore be considered as near to the original type of nutrition characteristic of a primitive flagellate.

The observation that photosynthesis in *Ochromonas* appears never to be sufficient for multiplication is worth further attention. The inefficiency is due perhaps to the small size and scanty pigmentation of the chromatophore. If that were so, other faintly-coloured organisms might be presumed to live in similar circumstances, for instance certain Myxophyceae (Geitler, 1925; pp. 148, 402; Pringsheim, 1949, pp. 61 et sqq.) or Chlamydomonadaceae (Pascher, 1927, p. 34)—instances where no experimental evidence is yet available. This, if verified, might shed light on the course of evolution. Did this proceed from originally fully phototrophic ancestors, so that reduction in photosynthetic capacity would be a step on the way towards full heterotrophy or did it start from primitive types which used their pigmented plastids only as a means of supplementing an original capacity to utilize organic substances?

The type of nutrition found in *Ochromonas* is peculiar and unexpected. The organism needs dissolved or enzymatically soluble carbon compounds as the main source of energy and building material; it needs in addition, as vitamins, certain compounds which it cannot synthesize, but it does not really need its pigment system (except for maintenance) once it has multiplied sufficiently so that a proportion of the cells are kept alive which then devour the weaker ones. What is so surprising in the nutritional behaviour of *Ochromonas* is that it has not been possible to obtain strict phagotrophy or phototrophy under experimental conditions, so that the nutrition is basically saprotrophic. One would have expected at least a slow multiplication to occur with the help of light energy alone, perhaps assisted by small amounts of certain vitamins supplied by food organisms. However, no appreciable multiplication took place under such circumstances, so that photosynthesis is probably very inefficient in *Ochromonas*. Phagotrophy therefore only supplements saprotrophic nutrition, as does also photosynthesis.

Diagnosis

Ochromonas malhamensis n.sp.

Shape of cell ovoid to spherical with a low depression in front where the flagella are inserted, but during movement often elongate, pear- to club-shaped, sometimes with a slight curvature. A pointed posterior end with a fine thread fastened to solid bodies may occur, but is much rarer than in other members of the genus. Spherical cells 6–8μ diameter; elongate cells 12 by 4μ.

The single chromatophore, consisting of two elongate portions connected by a bridge, changes much in size and coloration. It may be small and faint in colour, or large and golden brown. One contractile vacuole near the insertion of the flagella; this may be lacking. The longer flagellum may attain double the length of the body, while the short one is about one eighth of the other. Small individuals have relatively longer flagella. There is no eye-spot and no phototaxis. The reserve substances (leucosin (= chrysose), volutin, and oil) vary much in quantity.

The species is similar to *O. variabilis* H. Meyer (1897), from which it differs in not having two chromatophores. The description of this species does not cover the range of appearances exhibited by the new species, however.

The type strain was isolated in July 1949 from a rock in Malham Tarn (Yorkshire, England). Very similar organisms have been isolated several times in the U.S.A.

REFERENCES

Böcher, T. W., 1945. '*Ochromonas viridis* sp. n., a green flagellate, belonging to the Chrysomonadineae.' Vidensk. Medd. fra Dansk naturh. Foren., **108**, p. 233.

Bohlin, K., 1897. 'Zur Morphologie und Biologie einzelliger Algen.' Oefvers. K. Svensk. Vet.-Akad. Förhandl., p. 507.

Chadefaud, M., 1947. 'Une nouvelle Chrysophycée marine filamenteuse: *Nematochrysopsis roscoffensis* n.g. n.sp.' Bull. Soc. bot. France, **94**, 239.

Chen, Y. T., 1950. 'Investigations on the biology of *Peranema trichophorum* (Euglenineae).' Quart. J. micr. Sci., **91**, 279.

Doflein, F., 1916. 'Zuckerflagellaten.' Biol. Zentralbl., **36**, 439.

—— 1922*a*. 'Untersuchungen über Chrysomonadinen. I. *Ochromonas granularis* Dofl.' Arch. Protistenk., **44**, 149.

—— 1922*b*. II. 'Über *Chrysamoeba radians*.' Ibid., 206.

—— 1923. III. 'Arten von *Chromulina* und *Ochromonas* aus dem badischen Schwarzwald und ihre Cystenbildung.' Ibid., **46**, 267.

Fritsch, F. E., 1935. *The structure and reproduction of the algae, I.* Cambridge (University Press).

Gavaudin, P., 1931. 'Quelques remarques sur *Chlorochromonas polymorpha*, spec. nov.' Le Botaniste, Sér. **23**, 277.

Geitler, L., 1925. *Cyanophyceen. Süßwasserfl. Deutschl., Österr. u. d. Schweiz*, vol. 12 Jena (Fischer).

Hardin, G., 1942. 'An investigation of the physiological requirements of a pure culture of the heterotrophic flagellate, *Oikomonas termo* Kent.' Physiol. Zool., **15**, 466.

—— 1944. 'Physiological observations and their ecological significance. A study of the protozoan, *Oikomonas termo*.' Ecology, **25**, 192.

Huber-Pestalozzi, G., 1941. 'Das Phytoplankton des Süßwassers. Systematik und Biologie. 2. Teil, 1. Hälfte. Chrysophyceen, farblose Flagellaten, Heterokonten.' In *Die Binnengewässer*, Stuttgart (Thienemann).

Hutchens, N. L., Podolsky, B., and Morales, M. F., 1948. Cf. Hutner, S. H., and Provasoli, L., 1951. 'The Phytoflagellates,' in *Biochemistry and Physiology of Protozoa*, edit. by A. Lwoff, vol. i. New York (Academic Press).

Klebs, G., 1883. 'Über die Organisation einiger Flagellaten-Gruppen und ihre Beziehungen zu Algen und Infusorien.' Unters. Bot. Inst. Tübingen, **1**, 233.

Korshikoff, A. A., 1929. 'Studies on the Chrysomonads. I.' Arch. Protistenk., **67**, 253.

Mack, B., 1950. 'Morphologische und entwickelungsgeschichtliche Untersuchungen an Chrysophyceen.' Österr. Botan. Ztschr., **98**, 249.

Mainx, F., 1929. 'Untersuchungen über den Einfluß von Außenfaktoren auf die phototaktische Stimmung.' Arch. Protistenk., **68**, 105.

Mast, S. O., 1938. 'Digestion of fat in *Amoeba proteus*.' Biol. Bull., **75**, 389.

—— 1942. 'The hydrogen-ion concentration of the content of the food vacuoles and the cytoplasm in *Amoeba* and other phenomena concerning the food vacuoles.' Ibid., **83**, 173.

—— and Bowen, W. J., 1944. 'The food-vacuole in the Peritricha, with special reference to the hydrogen-ion concentration of its content and of the cytoplasm.' Ibid., **87**, 188.

Meyer, H., 1897. 'Untersuchungen über einige Flagellaten.' Revue Suisse Zool., **5**, 43.

Pascher, A., 1927. 'Volvocales-Phytomonadineae,' in *Süßwasserfl. Deutschl. Österr. u. d. Schweiz*, vol. 4, Jena (Fischer).

—— and Lemmermann, E., 1913. 'Flagellaten', in *Süßwasserfl. Deutschl. Österr. u. d. Schweiz*, vol. 2, Jena (Fischer).

Pringsheim, E. G., 1921. 'Zur Physiologie saprophytischer Flagellaten (*Polytoma*, *Astasia* und *Chilomonas*).' Beitr. allg. Bot., **2**, 88.

—— 1928. 'Physiologische Untersuchungen an *Paramaecium bursaria*.' Arch. Protistenk., **64**, 289.

—— 1935. 'Über Azetatflagellaten.' Naturwissenschaften, **23**, 110.

—— 1942. 'Contributions to our knowledge of saprotrophic algae and flagellata.' III. *Astasia, Distigmata, Menoidium*, and *Rhabdomonas*.' New Phytol., **41**, 171.

—— 1946*a*. *Pure cultures of algae*. Cambridge (University Press).

—— 1946*b*. 'The biphasic or soil-water culture method for growing Algae and Flagellata.' J. Ecol., **33**, 193.

—— 1948. 'The loss of chromatophores in *Euglena gracilis*.' New Phytol., **47**, 52.

—— 1949. 'The relationship between Bacteria and Myxophyceae.' Bact. Rev., **13**, 47.

—— 1950. 'The soil-water culture technique for growing algae.' Symposium of the Amer. Phycol. Soc., Christmas 1949 (New York).

—— and Mainx, F., 1926. 'Untersuchungen an *Polytoma uvella* Ehrbg., insbesondere über Beziehungen zwischen chemotaktischer Reizwirkung und chemischer Konstitution.' Planta, **1**, 583.

Schoenborn, H. W., 1950. 'Nutritional requirements and the effect of pH on growth of *Euglena viridis* in pure culture.' Trans. Amer. micr. Soc., **69**, 217.

Stosch, H. A. von, 1951. 'Über das Leukosin, den Reservestoff der Chrysophyceen.' Naturwiss., **38**, 192.

Wilber, C. G., 1942. 'Digestion of fat in the rhizopod, *Pelomyxa carolinensis*.' Biol. Bull., **83**, 320.

The Effects of Single Doses of Pilocarpine and of Histamine upon the Granules of Oxyntic Cells of Rats, with Special Reference to their Phospholipine Content

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With one plate (fig. 2)

Summary

1. New data are presented on the morphology and cytochemistry of the granules of the oxyntic cells of the rat's stomach in continuation of work previously reported (Menzies, 1949). The effects of single injections of pilocarpine and of histamine on the granules were investigated, with particular reference to their phospholipine content as shown by Baker's acid haematein method.

2. Pilocarpine causes a marked enlargement of the granules with loss of phospholipine from the central part of many of the larger granules, and complete loss of phospholipine from others. The granules that have lost their phospholipine probably still contain lipoid as shown by sudan black. Pilocarpine also causes marked aggregation of the granules.

3. Histamine causes a less marked increase in granule size and no observable loss of phospholipine, but some of the granules became elongated, an effect that can be seen with the phase-contrast microscope, and there is a tendency for them to recede to the peripheral parts of the cells associated with an enlargement of the intracellular canals.

4. Both pilocarpine and histamine result in some enlargement of the oxyntic cells, the effect being more marked with pilocarpine.

5. Neither pilocarpine nor histamine alters the size or shape of the non-lipoid moiety of the granules.

6. Supravital colouring with neutral red shows a few droplets in oxyntic cells in the bases of the gastric glands. These enlarge slightly after histamine and greatly after pilocarpine, and they are always spherical. It has not yet been possible to decide as to their exact nature.

Introduction

IN a recent paper (Menzies, 1949) it was shown that the granules of the oxyntic cells of the rat's stomach colour with sudan black and acid haematein (Baker, 1946), from which it was deduced that they contain lipoid, including probably phospholipine. It was also shown that the granules stain with iron haematoxylin after the removal of lipoids by prolonged extraction with hot pyridine, indicating a non-lipoid component, possibly of a protein nature.

In the present experiments the granules have been studied by a variety of cytological and histochemical methods after certain experimental procedures known to alter the activity of oxyntic cells in mammals. It was hoped by this

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means to ascertain if any change in shape, size, numbers, distribution or structure occurs in the granules, and possibly to throw light on their physiological significance. The work is a continuation of that already reported (Menzies, 1949). Accurate comparison of granule size is not possible because the smallest granules are near the limit of resolution of the light microscope.

Materials and Methods

In the following investigation male Norwegian rats averaging 200 gm. in body-weight were used. Because of the possibility that the number and size of the granules might be influenced by the physiological state of the cells, the control animals for the experiments listed below were fed (oatmeal, dried milk, and cabbage), placed in separate cages, and then starved for 24 hours before death, a procedure which experience showed gave a reasonably uniform picture. At the start of an experiment the rats to be used were similarly starved for 24 hours. During the period of starvation drinking water was freely available to all animals. Each rat was killed with coal gas.

In a few cases both vagi were severed in the neck under nembutal anaesthesia 3–5 days before the beginning of the period of starvation.

Solutions for injection were made up to 1 c.c. with distilled water and doses were calculated as milligrams per kilo body-weight.

Experiments with pilocarpine

Thirty animals received an intraperitoneal injection of 40 mg./kg. of pilocarpine nitrate, and were killed in pairs at intervals from 5 to 360 minutes afterwards. As the maximum effects on the oxyntic granules were obtained after 20–30 minutes, a further twelve rats were injected similarly and killed after 20–25 minutes.

Three rats were injected subcutaneously with atropine sulphate (0·3 mg./kg.) 10 minutes before the injection of pilocarpine and killed 25 minutes later. Two rats with severed vagi were injected with pilocarpine and killed 25 minutes later.

As controls two rats with severed vagi were killed after 24 hours' starvation. Two animals were injected with atropine sulphate alone. Two others were injected with distilled water, and these were killed 25–35 minutes later.

Experiments with histamine

Twenty rats were injected subcutaneously with histamine hydrochloride (5 mg./kg.) and killed in pairs at intervals from 5 to 120 minutes afterwards. The maximum effect on the granules was obtained after 45 minutes; so a further fifteen rats were injected and killed after 45 minutes. Three rats were given an injection of atropine sulphate (0·3 mg./kg.) 10 minutes before histamine, and killed 45 minutes after that.

As controls three rats were injected with distilled water and two with atropine and were killed 45–55 minutes later.

Histochemical and cytological techniques

Pieces of the mucous membrane from the fundus of the stomach were subjected to the following examinations:

A. *Tests for lipoids in general.* After fixation in formaldehyde-calcium and embedding in gelatin, frozen sections were coloured with sudan black (Baker, 1944), sudan II, sudan III, and sudan IV. A pre-war stock of sudan black gave satisfactory results but when this was exhausted new specimens of the dye proved unreliable; so two alternative methods devised for paraffin section were used (Baker, 1948; Thomas, 1948).

B. *Specific tests for lipines.* The acid haematein test (Baker, 1946), the most important method used so far as this present paper is concerned, was applied (before and after the extraction of the lipoids with hot pyridine). Sudan black was also applied after hot pyridine in order to confirm the removal of all lipoids. A positive reaction to the acid haematein test indicates the presence of a phospholipine (Baker, 1947*a*). After lipoid extraction some sections were cut in wax at 5 μ and stained with Heidenhain's iron haematoxylin to demonstrate the non-lipoidal component of the granules.

C. *Miscellaneous tests.* Tyrosine (Millon's reaction) and arginine, &c. (Baker, 1947*b*), were tested for in the hope of elucidating something of the structure of the non-lipoidal component of the granules.

As the investigation might involve the comparison of oxyntic granules with both mitochondria and Golgi material the following methods were used:

D. *For mitochondria.* Janus green B and janus black (1/10,000 at 37° C. for 10 minutes) were employed supravitally.

Altmann's acid fuchsin (with nuclear staining by methyl green) was used after Regaud fixation.

Pieces of unfixed mucosa were examined in normal saline with the phase-contrast microscope.

E. *For Golgi material.* Neutral red chloride B.D.H. (1/10,000 at 37° C.) was employed supravitally.

Aoyama's silver method and the Nassonov-Kolatchev osmium textroxide method were also used.

To describe the increase in size of cells following pilocarpine and histamine the longest diameters from camera lucida drawings of one hundred of the largest cells seen in the acid haematein preparations in each group were measured, and an average taken.

Results

A. *Where tests for mitochondria were applied*

Ordinary and phase-contrast microscopy showed that the oxyntic cell cytoplasm in all animals was filled with numerous granules of varying size. The chief result following the injection of pilocarpine and histamine was the presence of some very large granules bigger than any seen in the normal cells, and pilocarpine produced larger granules than histamine. Elongation of

granules was present in some cells only after histamine; otherwise they were all spherical and probably equally numerous in all animals. In normal animals janus green B and janus black coloured the granules green and black respectively, and the Regaud-Altmann technique (fig. 2A) stained them crimson against a yellowish background. Whereas they were all spherical with the former dyes, the latter technique showed also many short rods. These showed no particular orientation and were not confined to any one part of the cell.

B. *For Golgi material*

Supravital neutral red showed a small number of the oxyntic cells (normal and experimental) in the deepest parts of the gastric glands contained a few red droplets. Many were larger than normal after histamine and still larger following pilocarpine. The droplets were distributed at random, and the majority of the oxyntic cells contained no such droplets.

In normal oxyntic cells the Nassonov-Kolatchev technique showed either (*a*) both osmiophil granules and strands in the cytoplasm, (*b*) a definite osmiphil network throughout the cytoplasm, or (*c*) a localized collection of dark osmiphil strands. After histamine the majority showed larger grey indistinct granules and a few showed osmiphil rings around large clear central areas. Possibly these rings surround several granules that have coalesced. In normal animals and following histamine, 'typical Golgi nets' were seen in non-oxyntic cells. In all animals after pilocarpine the oxyntic cells showed numerous small jet-black granules, but as similar ones appeared in the connective tissue, and sometimes even red blood cells were impregnated, and, moreover, as typical Golgi nets were not observed in other cells, perhaps pilocarpine in some way interferes with the impregnation.

The results with Aoyama's technique were similar in normal and experimental animals. The granules in the majority of the oxyntic cells were not impregnated by the silver. A few cells showed a number of blackened particles, and a very few showed a localized area of impregnated granules around one pole of the nucleus. None of these pictures resembled the cord-like Golgi nets seen in peptic and other cells in the same sections.

C. *For the lipoidal components of the granules*

A single injection of pilocarpine or histamine resulted in a general increase in size of the oxyntic cells as compared with the normal. After pilocarpine the maximum size was reached in 20–30 minutes when they averaged 26·0 μ as compared with 18·6 μ for the normal. After 30 minutes the cells decrease in size, but even after 6 hours they were still larger than normal. With histamine the increase in cell size was slight. An average maximum of 20·1 μ was reached in 35 minutes; the size returned to normal after 120 minutes.

In the normal animals all the oxyntic cells were filled with spherical sudanophil granules similar in relative size and distribution to those seen in the living cell. The largest had approximately twice the diameter of the

smallest and there were granules of intermediate size present. After both histamine and pilocarpine, sudan black and acid haematein showed that there was an increase in granule size. With pilocarpine (figs. 1B, 2C) the maximum size was reached 20–30 minutes after injection, when the largest had approximately twice the diameter of the largest seen in the normal (figs. 1A, 2B). With histamine, the granules were never so large as after pilocarpine: they

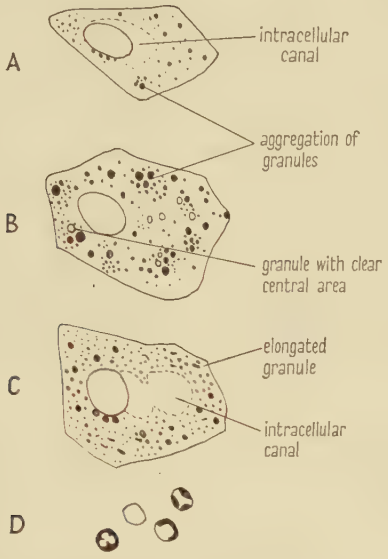

FIG. 1. Freehand drawings of typical oxyntic cells (acid haematein test). A, normal; B, 25 minutes after pilocarpine; C, 45 minutes after histamine; D, typical oxyntic granules that showed uncoloured central areas with acid haematein.

reached their maximum size (figs. 1C, 2D) in 45 minutes and were indistinguishable from normal again in 90–120 minutes. With pilocarpine they were still larger than normal even after 6 hours. After either drug there were many granules of lesser size present although very few were as small as the smallest seen in the normal. Aggregation of granules was observed in a few cells in all the normal animals, and was a marked feature in many cells after pilocarpine (fig. 1B), the maximum effect being shown 20 minutes to 3 hours after injection. Granule aggregation was only occasionally found after histamine, being less marked than in the normal.

In the twenty-four normal rats studied, most of the granules were coloured intensely with acid haematein (fig. 2B), when they appeared as uniformly black or blue-black spheres, no internal structure being visible. After pilocarpine many of the largest granules in some of the cells showed uncoloured central cores with acid haematein (fig. 1 B and D) but not with sudan black. Usually these granules appeared as a peripheral black ring of uniform thickness surrounding an uncoloured circular medullary area. In many, however, the black peripheral ring appeared beaded as if the coloured material were clumped at

points along its circumference. In a few granules the peripheral ring coloured by acid haematein was much thicker in one half of its circumference than in the other half, and very occasionally the uncoloured medulla appeared trifoliate (fig. 1D). Except for one rat killed 10 minutes after pilocarpine these phenomena were seen only 20–30 minutes after injection, and were never observed in normal cells or after histamine. The rod-shape of some of the granules observed in the living cell after histamine was confirmed with acid haematein (fig. 1C), but was never seen in normal or pilocarpinized animals. The intensity of colouring with acid haematein was more variable after pilocarpine and histamine than in the normal, for whereas in the normal the granules which coloured were blue or blue-black in the experimental animals in one and the same cell some granules appeared black, and some grey or blue.

In a few deeply seated cells, in about 50 per cent. of the normal animals investigated, some of the granules remained uncoloured with acid haematein (but not with sudan black), though they were still visible by virtue of their refractility. This occurred in one rat only after histamine, but in all following pilocarpine. Although never marked, there were more of these 'negatively reacting cells' present in each of the fourteen rats killed 20–30 minutes after pilocarpine than in any of the normals. Intracellular canals were frequently clearly visible in acid haematein preparations of normal rats. They were not appreciably different from those of normal in rats killed from 3 to 6 hours after injection of pilocarpine, but in animals killed after shorter intervals they were less obvious than in the normal. On the other hand, a noticeable effect of histamine was inflation of some of these canals (maximal after 35 minutes) and in a few cells this swelling of the canals led to the granules taking up a peripheral position in the cell, an effect not observed in the normal or after pilocarpine.

Neither severance of the vagi, nor a previous injection of atropine (known to paralyse the vagal nerve endings), affected the results with histamine; and when these procedures were carried out before an injection of pilocarpine, sections were indistinguishable from normal. Control animals in which neither pilocarpine nor histamine were injected were also unaffected by these procedures, and by the injections of distilled water alone.

Sudan black did not colour the oxyntic granules after extraction of lipoids from the mucosa with hot pyridine.

The results with the remaining tests concerned only the non-lipoidal components of the granules and were as follows:

After the extraction of lipoids with pyridine, the oxyntic granules stained a dark brown with iron haematoxylin. These granules were numerous, of varying sizes, and stained homogeneously. No difference could be seen between

FIG. 2 (plate). A, typical picture of oxyntic granules as shown by the Regaud–Altmann technique. B–D, photographs of frozen sections (10–15 μ) coloured by acid haematein, for comparison of granule-size. B, normal; C, after pilocarpine; D, after histamine. Note that in C some oxyntic cells are visible in which the granules no longer colour with acid haematein.

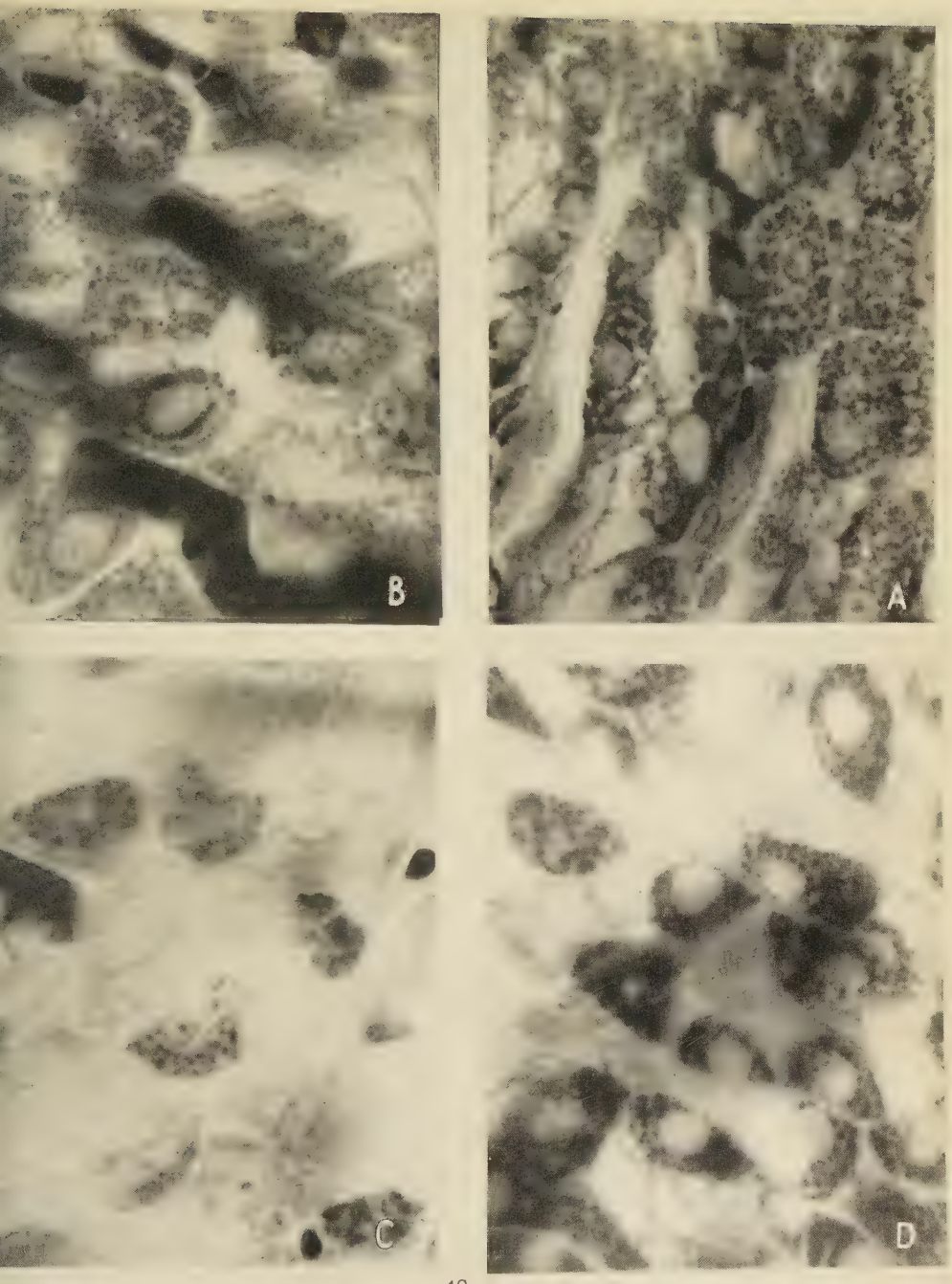

FIG. 2

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normal and experimental animals, this being in striking contrast to the results given by acid haematein and sudan black. Most of the granules gave a positive result in tests for certain guanidine derivatives (e.g. arginine), and also for tyrosine, the latter substance being demonstrated in the general cytoplasm as well as in the granules.

Discussion

The present investigation adds to what has already been reported (Menzies, 1949) about the morphology of the oxyntic granule in the 'normal' rat (i.e. after starvation for 24 hours). The granules are clearly visible in the living cell by phase-contrast microscopy, and a few are elongated, but only following injection of histamine. They stain supravitally with janus green B, as already reported by Lim and Ma (1926) in the rabbit, and with janus black. Supravital neutral red shows a few red droplets in basally situated oxyntic cells as already observed by Dawson (1948) and the present results show that these enlarge after either pilocarpine or histamine. It is not possible yet to determine whether these red-coloured droplets are separate vacuoles which might be comparable with the vacuolar system of a Golgi element, or whether they are stained oxyntic granules, as claimed by Dawson, who, however, produced no definite evidence to support this view. The present results show that the non-lipoid component of the oxyntic granule contains protein and guanidine or its derivatives. It has so far not been possible to demonstrate typical mitochondria or a recognizable Golgi element in the oxyntic cells.

Both pilocarpine and histamine, in single injections, produce definite alterations in the size and structure of the granules, the former producing the more marked changes. Neither pilocarpine nor histamine produces any alteration in the size of the non-lipoid component of the granules. Pilocarpine produces enlargement of the granules, as shown by acid haematein, with the appearance of non-lipine central cores in many granules, and complete loss of lipine in others. Histamine also produces enlargement of the granules, some of which become elongated; it does not result in the appearance of non-lipodial central cores. A striking effect of histamine in some cells is an enlargement of the intracellular canals. The effects of pilocarpine are evidently mediated by the vagal nerve endings and acetylcholine, for they are abolished by previous section of the vagi and by atropine. The effect of histamine is direct, for these procedures do not alter its action on the granules.

Considering the results already described it is tempting to discuss the structure and function of the oxyntic granules and the fact that they may play some role in the secretion of hydrochloric acid. However, work at present in progress makes it unadvisable to enter into a more detailed discussion at the moment, and these points will be considered at a later date. It may be as well to stress, however, that although Fulton (1949) denies that histamine produces acid secretion in the rat, nevertheless the changes in the granules, as shown by acid haematein after a single injection of histamine, are always observed in this animal.

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REFERENCES

Baker, John R., 1944. Quart. J. micr. Sci., **85**, 1.
—— 1946. Ibid., **87**, 441.
—— 1947*a*. Ibid., **88**, 115.
—— 1947*b*. Ibid., **88**, 463.
—— 1948, published in article by O. L. Thomas (1948).
Dawson, A. B., 1948. Anat. Rec., **100**, 319.
Fulton, J. F., 1949. *A textbook of physiology*. 16th edition. Philadelphia and London (Saunders).
Lim, R. K. S., and Ma, W. C., 1926. Quart. J. exp. Physiol., **16**, 87.
Menzies, G., 1949. J. Anat., **83**, 25.
Thomas, O. L., 1948. Quart. J. micr. Sci., **89**, 333.

The Role of Iron in Histological Staining

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With two plates (figs. 1 and 2)

SUMMARY

Rapid methods for staining sections with iron sulphide are described which give results comparable with iron haematoxylin.

Nucleic acids and proteins are mainly responsible for the uptake of iron. The staining capacity of different proteins runs parallel with the number of free carboxyl groups they contain. It is concluded that the iron forms non-ionizing complexes with carboxyl and phosphoric acid groups.

The intensity of staining is a measure of the relative abundance of such groups in the proteins and nucleic acids present, and of the concentration density of these substances.

In the staining of nuclei and chromosomes with iron the proteins are at least as important as the nucleic acids.

The iron taken up is mostly in the ferric state but it is partially converted to ferrous iron by reducing substances (chiefly sulphydryl groups) present in many fixed tissues. Iron in the form of iron sulphide or undifferentiated iron haematoxylin therefore acts as a metachromatic stain: a blue colour indicates ferrous iron, a brown colour ferric iron.

IT has long been known that nuclear structures have a high affinity for iron. The iron which A. B. Macallum (1908) believed to be a normal constituent of 'nuclein' was shown by Gilson (1892*a*) and by Wiener (1916) to have been picked up during the manipulations from the traces of this metal present in the vessels and reagents used. The absence of notable quantities of iron in normal nuclei has been confirmed by micro-incineration (Policard, 1934).

Gilson (1892*b*) made use of this affinity for iron in devising a 'chemical method' for staining nuclei and he describes a somewhat elaborate mixture of iron and nickel salts which gives rather pale staining of nuclear structures. Iron sulphide had been used much earlier by Landois (1865) and Leber (1868), but no differential staining of nuclei was claimed and later reports have been unfavourable (Gierke, 1884).

The present work, which was started with the object of reinvestigating the possibilities of this type of metallic staining, has led to a consideration of the part played by metals, and particularly by iron, in histology. Before dealing with these matters it will be well to describe the methods that have been devised.

Sections were immersed in solutions of the salts of metals which have black insoluble sulphides, rinsed well in running water, and immersed in distilled

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water to which a little ammonium sulphide had been added. Silver, lead, &c., gave completely negative results; copper gave almost no staining; nickel and cobalt were a little better and did give some nuclear staining; but iron was in a class by itself and gave excellent staining of the nuclei.

Techniques for Staining with Iron Sulphide

The procedures finally adopted were as follows:

(i) The sections are immersed in a saturated solution of iron alum for 1 minute, washed well in running water, and immersed in dilute ammonium sulphide for 15 seconds or longer.

Results: nuclei blue-black; chromosomes intense blue-black; cytoplasm grey or brown; fibrous tissue pale chocolate brown; Malpighian layer of mammalian skin blue; red blood corpuscles pale blue-grey. Fig. 1A shows a whole mount of resting and dividing cells in the fat body of the insect *Rhodnius* stained with iron sulphide alone.

If the staining is too dark it may be differentiated by immersing for a few seconds in 0·25 per cent. iron alum. The section of the heart, epidermis, and cuticle of *Rhodnius* in fig. 1D was prepared in this way. Alternatively, after the initial immersion in iron alum the washed section may be rinsed in 0·1 per cent. nitric acid for a few seconds before transfer to the ammonium sulphide.

The amount of iron taken up by a given section is constant and never exceeds a certain maximum. Since iron sulphide is soluble in iron alum it is not possible to obtain deeper staining by repeating the process; but where heavier staining is required the following procedures can be used.

(ii) After immersion in ammonium sulphide the section is transferred to a solution of potassium ferricyanide in which the iron sulphide is at once converted to Turnbull's blue. This is insoluble in iron alum; consequently the staining process can be repeated and on immersion a second time in ammonium sulphide a double quantity of iron sulphide is formed. This procedure can be repeated to give the desired depth of staining. Fig. 1E shows a section of an early rabbit embryo deliberately overstained in this way.

(iii) An alternative method of obtaining darker staining consists in transferring the section, after treatment with ammonium sulphide and washing in water, to a saturated solution of cuprammonium sulphate. Within a second or

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Fig. 1

A. Dividing cells in fat-body of *Rhodnius* (whole mount). Iron alum, ammonium sulphide.

B. Sebaceous gland above and sweat gland below, from human scalp. Iron alum, ammonium sulphide, cuprammonium sulphate, ammonium sulphide.

C. Stomach of cat. Staining as in B.

D. Heart with pericardial cells inside, fat-body, epidermis, and cuticle of *Rhodnius*. Iron alum, ammonium sulphide, differentiation in dilute alum.

E. Neural tube in early rabbit embryo. Iron alum, ammonium sulphide, potassium ferricyanide (cycle repeated three times), iron alum, ammonium sulphide.

F. Gill of *Ostrea*. Iron alum, ammonium sulphide, nickelammonium chloride, ammonium sulphide.

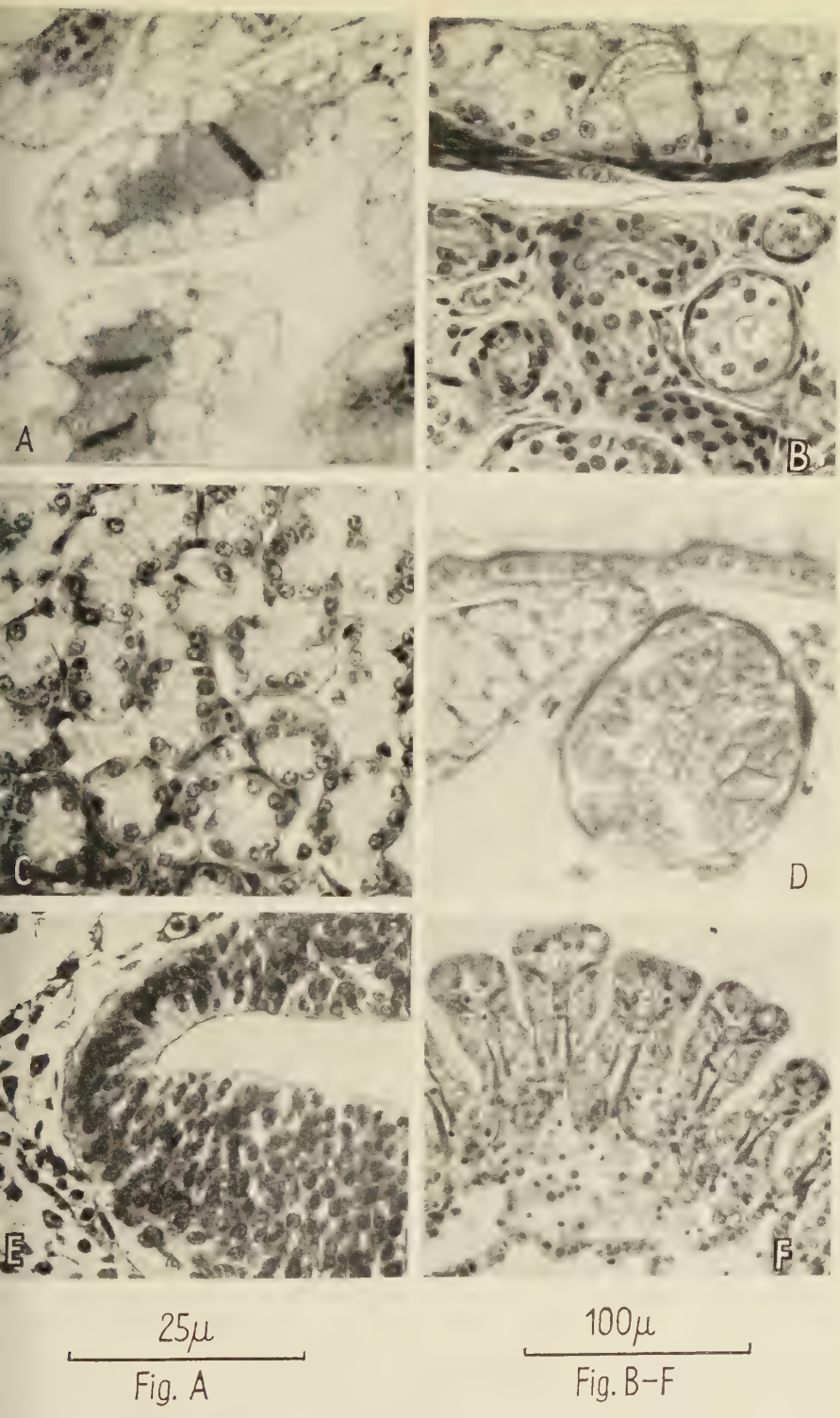


Fig. 1

V. B. WIGGLESWORTH

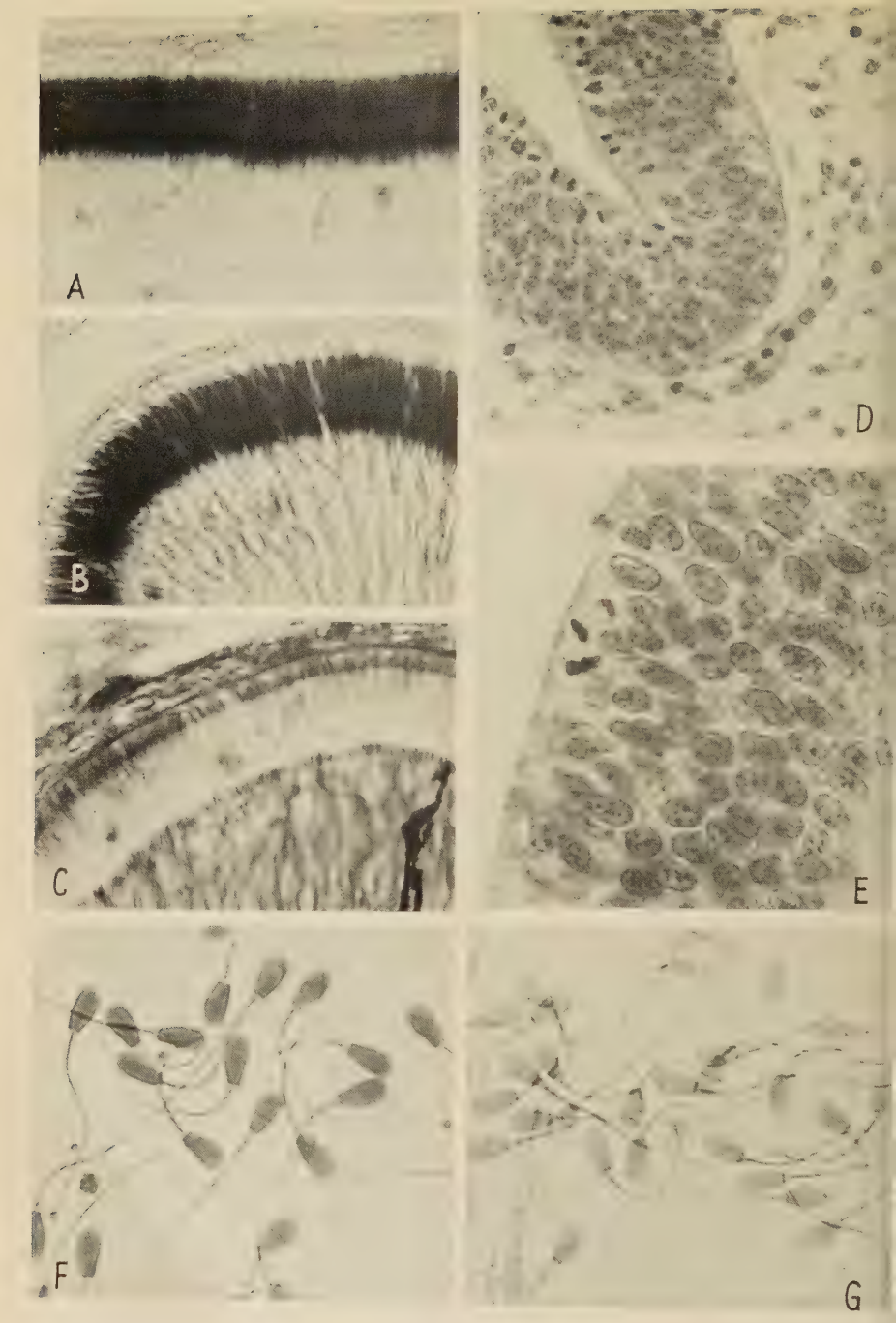


Fig. 2

wo the iron is displaced by the copper to give the rather pale brown copper ulphide. But the iron is precipitated *in situ* as the hydroxide so that if the sec- ion is rinsed in water after half a minute and re-immersed in the ammonium ulphide, deposits of both iron and copper sulphide are thrown down. This ives a very agreeable sepia colouring well suited for photography. Fig. 1C, howing the stomach of the cat, and fig. 1B, showing parts of a sweat gland nd sebaceous gland in the human scalp, were prepared in this way.

The same procedure can be used with any metal which forms a soluble mmine complex with ammonia and is more electronegative than iron. Cobalt nd nickel displace the iron more slowly than copper; silver acts rapidly but he ammoniacal solution is unstable, whereas the other salts can be used re- eatedly. Cuprammonium sulphate or nickelammonium chloride are the most enerally useful. Fig. 1F shows the gill of *Ostrea* prepared with nickel- mmonium chloride.

The sulphides of cobalt and nickel are not soluble in strong solutions of the arent salt; by repeated transfer between saturated cobalt nitrate or saturated ickelammonium chloride and the ammonium sulphide solution it is possible o build up the grey-brown staining of the tissues to any required depth.

Fixatives and Mountants

Most ordinary fixatives can be used. Carnoy (fig. 1, A and D), Bouin (B and C), Heidenhain's 'Susa' (E and F), Zenker's or Helly's mercuric chloride and otassium dichromate have all given good results. Osmium tetroxide inter- eres with the subsequent uptake of iron; Flemming's or Altmann's mixtures do not give satisfactory results.

Iron sulphide is readily oxidized in the presence of acid so that the staining ades rapidly in many samples of Canada balsam. This may be prevented by dding a little butylamine to the mountant, and such preparations have re- mained unchanged for 18 months—although the medium gradually darkens. Euparal with the addition of butylamine also gives permanent preparations. Thick cedar-wood oil ringed with Hazen's cement has been much used; fig. 1A was photographed a year and a half after mounting in cedar-wood oil. Gurr's neutral mounting medium, mounting being made directly from absolute alcohol, has proved the most generally useful (fig. 2, A–E).

Fig. 2

A. Closely packed spermatozoa in median vesicula seminalis of *Lumbricus*. Feulgen's method.

B. The same. Iron alum, ammonium sulphide.

C. The same. Extraction with 5 per cent. trichloracetic acid at 90° C.; iron alum, ammonium sulphide.

D. Neural tube of early rabbit embryo. Extraction with 5 per cent. trichloracetic acid at 90° C.; iron alum, ammonium sulphide.

E. The same, more highly magnified.

F. Spermatozoa of bull. Normal; iron alum, ammonium sulphide.

G. Spermatozoa of bull. Extraction with 5 per cent. trichloracetic acid at 90° C.; iron alum, ammonium sulphide.

Factors Influencing the Uptake of Iron

Many factors influence the amount of iron taken up by the sections. Th following have been considered.

(i) *Valency*. Ferric iron is taken up much more rapidly than ferrous. M ferric ammonium sulphate was compared with freshly prepared M/2 ferrou sulphate, sections of rat embryo at 8μ being used as test material. In the cas of the ferric salt uptake appeared to be complete within about 1 second; then was no perceptible difference in the amount of iron taken up after immersio for 1 minute and 1 second. In the case of the ferrous salt very little iron wa taken up in 5 seconds. The amount increased as immersion was prolonged bu it did not reach its maximum for several hours. After immersion for 12 hour the depth of staining was about equal to that given by the ferric salt.

(ii) *Time of immersion*. As seen in (i) the uptake of ferric iron is complet within a second or so. If treatment in saturated iron alum is prolonged fo 2 days staining is very slightly darker than after immersion for 15 seconds when both preparations are examined at once. When examined a week late the difference is more marked; the staining is more permanent in the section given 2 days' immersion in the alum. (Perhaps, after very brief immersion there are some free acid groups remaining which lead to partial fading of th sulphide.)

(iii) *Concentration*. Dilution of molar ferric chloride in distilled water t M/5 causes a reduction in the depth of staining equal to perhaps 10 per cent Dilution to M/25 causes a further reduction of about 10 per cent. Staining becomes progressively less at greater dilutions, though even at M 30,000 some iron is absorbed. At each concentration there is a maximum uptake which is soon reached and is not changed by prolonged immersion.

(iv) *Temperature*. The effect of temperature is complicated by hydrolytic dissociation; the solutions become darker on warming owing to the increased formation of ferric hydroxide. Ferrous salts are much less subject to hydrolytic dissociation. Freshly prepared ferrous sulphate (M 2) gives a greater speed o uptake at 80° C. than at 20° C. but the depth of staining which is finally reached is unchanged. It would appear that high temperature accelerates the uptake but has no effect on the final equilibrium.

(v) *Acidity*. We have seen (p. 106) that iron is readily removed from the sec tion by treatment with dilute acids before immersion in ammonium sulphide The uptake of iron is also depressed by increasing acidity. With a given concentration of iron salt the depth of staining is reduced as the pH is lowered but with high concentrations (M $2FeCl_3$) all the constituents of the tissues will stain as usual in N.HCl, although less strongly than in neutral solution These results have been obtained equally with ferrous iron in which little hydrolytic dissociation occurs. The effect of acidity on staining will be considered in more detail later (p. 113).

These results do not provide conclusive evidence of the nature of the forces by which iron is held in the tissues; but the effects of valency, of temperature,

nd of acidity suggest that the metal is held by chemical combination rather aan by physical adsorption.

Substances Responsible for Binding Iron

The iron taken up by tissue sections is in the 'masked' state. If the sections ter immersion in iron alum are washed in a saturated aqueous solution of ppper sulphate, only a very small part of the iron is displaced by copper. ections after treatment with iron alum give almost no colour with neutral otassium ferrocyanide, and sections after treatment with ferrous sulphate ve no colour during brief immersion in ferricyanide. Clearly the iron is in me non-ionizing complex, but this complex is readily broken down by mmonium sulphide.

Many biological substances form complexes with iron (Tompsett, 1934), ut only those which are non-diffusible and which are present in relatively assive concentration will bind enough iron to be visible in histological sec-ons. The obvious materials in question are nucleic acids, proteins, perhaps rbohydrates and phosphatides.

Nucleic acids. If nucleic acid is dissolved in a saturated solution of urea and little ferric chloride is added, nucleic acid is precipitated and carries the iron wn with it so firmly bound that it will give no colour with ammonium thio-anate (Smythe and Schmidt, 1930). If finely powdered nucleic acid is dusted a microscope slide lightly smeared with glycerine-albumen and held in ace with a thin film of celloidin, it can be subjected to the same staining pro-dures as the tissue sections. Immersed in iron alum, rinsed in water and ppper sulphate, and treated with ammonium sulphide, each particle shows thin black coating over a colourless interior. Apparently the precipitate on e surface prevents the iron from reaching the centre of the particle. If the ucleic acid is very finely dispersed by allowing the solution in urea to dry on e slide and then covering with celloidin, an intense general staining with the on is obtained. If the nucleic acid before drying and powdering is mixed ith a non-staining protein like salmine (p. 110), this seems to facilitate the try of the iron and the particles stain throughout.

The importance of nucleic acids in iron staining cannot be in doubt. The rt they play has been studied in sections of *Lumbricus*. If these are stained by eulgen's method after Heidenhain's 'Susa' fixative the abundance of desoxy-onucleic acid in the developing spermatozoa is very striking. In the young ermatids it is in the form of dense granules in the nuclei; later it forms a und homogeneous core to the spermatid with a non-staining cortex. The re gradually elongates to form the head of the spermatozoon and when the e sperm come to line the median seminal vesicle their closely packed heads pear in the Feulgen-stained sections as intense red bands (fig. 2A).

If the sections are stained with iron sulphide, the most strongly Feulgen-sitive structures stain an intense blue-black; the structures showing a weaker eulgen reaction stain a paler blue-grey; most of the remaining structures in varying shades of grey-brown (fig. 2B). In the epidermal and some other

cells there are blue-grey zones around the nuclei, and in the nerve-cells ther are blue-grey networks. The nucleoli stain blue-black. Ribonucleic acid presumably responsible for much of this deep staining.

If the sections are heated in 5 per cent. trichloracetic acid at 90° C. fo 15 minutes (Schneider, 1945) to remove the nucleic acids, and are then staine with iron sulphide, most of the blue-grey and blue-black structures will dis appear. The bands of ripe sperm show an almost colourless zone where th deeply staining heads were before (fig. 2C). The core of the spermatids unstained and the nucleoli in the giant nerve-cells and in other cells ar markedly paler.

Proteins. In many tissues, however, the extraction of the nucleic acids wit trichloracetic acid makes comparatively little difference to the staining wit iron. In the placenta and embryo of the rabbit, for example, there is almost n reduction in the depth of staining in the nuclei and chromosomes after th nucleic acids have been removed (fig. 2, D and E). Clearly the residual protein i the chromosomes is largely responsible for the uptake of iron. (Kaufmann *et a* (1950) showed that the protein framework of the chromosomes stains with aci dyes even better than normally, after removal of nucleic acids.) In the saliva chromosomes of *Drosophila*, although there is some reduction in iron stainin after trichloracetic acid extraction, their normal banding can still be clearly see

The question arises whether the differential staining of the tissues after re moval of nucleic acid is due in part to differences in the capacity of differe proteins for binding iron or whether the intensity of staining is simply measure of the density of the protein.

This has been studied by dusting finely powdered proteins on to micr scope slides thinly smeared with glycerine-albumen, covering with a very thi layer of celloidin, denaturing in alcohol, and staining by the same procedu as the tissue sections. It is convenient to apply four different proteins to fou small quadrants on the slide so that they receive identical treatment. Th depth of staining varies widely, of course, with the size of particle, but it is no difficult to choose for comparison particles of the same size—a few micra i diameter. The method is highly subjective and will only give reliable result where there are large differences in the affinity for iron.

Table 1 summarizes the results. The staining properties differ widely. Th most deeply staining are the muscle proteins tropomyosin and myosin fro the rabbit; the nuclear proteins from the thymus of the calf, chromosomi and histone, fall little short of these; whereas the cereal seed proteins, zein an gliadin, and the protamine salmine are virtually unstained. Clearly there ar large differences in the affinity for iron in the different proteins.

In the spermatozoa of *Lumbricus* after extraction with trichloracetic acid th sperm head is quite colourless in the posterior half; it shows a very sligh grey-brown staining in its anterior half. This difference is just visible in fig. 2 but is conspicuous in the specimens. The deep blue-black staining of th normal sperm head is clearly due wholly to nucleic acid; the protein moiet must be almost all of the non-staining, probably protamine, type. On th

other hand, the apical piece and the middle piece stain quite deeply after removal of nucleic acid (fig. 2C). It is otherwise in the spermatozoa of the bull. Fig. 2F shows a smear of normal bull sperm fixed with Bouin's solution and stained with iron sulphide. Fig. 2G shows a similar film after trichloracetic acid extraction. The head after extraction is much paler (it now stains less than the tail) but it still retains a considerable amount of iron. Perhaps the protein here is of the histone or chromosomin type.

TABLE 1

Protein	*Free anions (per cent.)*	*Notes on iron sulphide staining*
Tropomyosin	26·6	Intense dark staining.
Myosin	18·0	Intense dark staining.
β-lactoglobulin	18·5	Deep staining.
Ovalbumen	13·9	Deep staining (slightly less than lactoglobulin).
Edestin	13·5	Deep staining (slightly more than ovalbumen).
Chromosomin (thymus)	..	Deep staining (slightly more than ovalbumen).
Histone (thymus)	..	Deep staining (a little less than chromosomin).
Collagen	10·7	(Fibrous tissue stains strongly.)
Insulin	9·3	Very pale staining.
Wool keratin	7·2	Variable staining; difficult to compare.
Silk fibroin	2·78	Uniform rather pale grey staining.
Zein	0·63	Very pale staining.
Gliadin	0·0	Virtually unstained.
Salmine	0·0	Virtually unstained.

Other substances. Grains of 'soluble starch' retain little iron. They give a uniform pale staining, showing that the iron penetrates rapidly throughout the grain, but very little is held. The chitinous cuticle of insects shows no more staining than can be expected from its protein content. Carbohydrates are probably of little importance in the uptake of iron. Egg lecithin smeared on a slide will bind considerable amounts of iron; phosphatides firmly held by the tissue proteins may play a small part in staining with iron. Guanosine phosphoric acid will bind iron like nucleic acid; the various mononucleotides present in the tissues may contribute to its retention, and so doubtless do other non-diffusible phosphoric acid compounds.

Nature of the Union with Iron

There seems little doubt that iron will be bound by any monesters or diesters of phosphoric acid present in the fixed tissues. It was shown by Fischer and Hultzsch (1938) that all such esters will form non-ionized compounds with ferric iron. They consider that the combination with nucleic acid is of this type. Whether these compounds are simple salts or chelate complexes is uncertain. A precipitate will form with diphenylphosphoric acid, for example, where there is little likelihood of chelation. In the case of nucleic acids, however, it would seem that there are many opportunities for the formation of co-ordinate linkages to stabilize the electrovalent bond—for example, with the hydroxyl groups of the sugar.

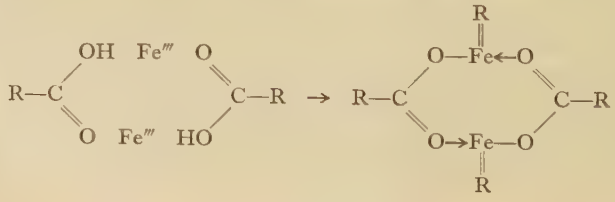
The binding of iron by proteins has been the subject of much argument. Bechhold (1928) and his co-workers found that a constant equivalent of iron was firmly held by protein and could not be washed out, and that equivalent quantities of the metal and its anion were retained. They concluded that the salt was held in combination by electrostatic adsorption to the amino and carboxyl groups of the protein. Other authors have favoured the view that there is chemical combination between the iron and the protein. As Fischer and Hultzsch (1938) point out, the matter can only be proved when a stoichiometric relation has been demonstrated between the amount of iron in combination and the concentration of some active group in the protein.

It is possible, however, to get some indication of the probable nature of the linkage from the staining experiments. In the first place the free amino groups would seem to play no part. If powdered egg albumen dusted on a slide (or sections of tissues) are soaked in 40 per cent. formalin so as to block the free amino groups and then immersed in equal volumes of formalin and the iron alum solution, there is not the slightest reduction in the amount of iron retained.

On the other hand, if the free carboxyl groups are methylated by immersion for two or three days in methyl alcohol containing N/10 hydrochloric acid (Fraenkel-Conrat and Olcott, 1945), the uptake of iron is markedly reduced. Finely powdered proteins (ovalbumen, chromosomin, histone sulphate) fixed to microscope slides were treated in this way, transferred to 5 per cent. ferric chloride in acetone for half a minute, rinsed in water, and then immersed in ammonium sulphide. Compared with non-methylated proteins similarly stained there was a striking reduction in the quantity of iron taken up. There was a similar great reduction in the depth of staining of the tissues.

Different proteins vary widely in the number of free carboxyl groups which they contain. Table 1 shows the percentage of free carboxyl groups among the side chains of the proteins studied, taken from the review by Tristram (1949). It can be seen that there is general agreement between the affinity of a given protein for iron and the number of free carboxyl groups in the molecule. The only serious exception for which data exist is insulin, which would have been expected to stain much more deeply than it does. The other polar side chains, amino, amide, phenolic, hydroxyl, sulphydryl (as set out in Tristram's review) show no correlation with the intensity of iron staining.

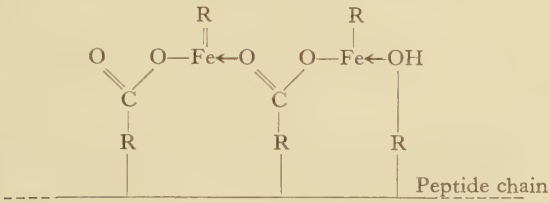
In the binding of iron by protein it thus seems highly probable that the initial linkage is an electrovalent bond with free carboxyl groups. But the stability of the complexes of iron with organic compounds depends upon the tendency for iron to form chelate compounds of the type



which the ionization of the salt linkage is suppressed by the co-ordination f the iron atom with some adjacent group. Ferric iron seems to have a special ndency to link through oxygen. Indeed, the affinity of iron for carboxyl roups is so great that calcium oxalate will actually dissolve in solutions of rric chloride (Sidgwick, 1950). Ferrous iron will also form complexes of this pe but does so much less readily.

It was shown by Smythe and Schmidt (1930) that ferric iron will form non-nizing complexes with carboxylic acids most readily when there is another rboxyl group or a hydroxyl group immediately adjacent to the first carboxyl. or example, lactic acid or citric acid are effective, α-hydroxybutyric acid is uch more effective than β-hydroxybutyric, whereas there is little tendency form such complexes with α-amino acids.

In the case of proteins, therefore, it seems probable that the suppression of nization of the salt linkage is brought about by co-ordination of the iron ith some neighbouring group; perhaps the double-bonded oxygen of another rboxyl group, perhaps more probably the oxygen of an adjacent hydroxyl roup.



The formation of such compounds must, of course, depend ultimately upon e steric possibilities of the molecules in question, a matter which has not en critically considered. The same problems arise in connexion with the ordanting of wool preliminary to dyeing. Although the nature of the union chromium and other metals with the fibre is not exactly known it is believed the present time to be of the same general type as that outlined above (Race, we, and Speakman, 1946; Bird, 1947).

Differential Staining of Proteins and Nucleic Acids

In acid solution iron will be displaced from its salt linkages by hydrogen d the complexes will break down. This will occur more readily with the aker carboxylic acids than with the stronger phosphoric acids. It is there-e to be expected that at low pH iron will be more firmly retained by those ments in the tissues that are most rich in nucleic acids and phosphoproteins. deed, Fischer and Hultzsch (1938), working with very dilute ferric chloride H/5000), showed that the binding of iron by isolated constituents of the sues under strongly acid conditions is due wholly to phosphoric acids. The ximum uptake of iron by nucleic acids is attained at pH 1·5, whereas the take by proteins continues to increase up to pH 4 or higher.

It was hoped that this might afford a means for the differential staining of oteins on the one hand, and nucleic acids and phosphoproteins on the

other; but the experiment did not prove wholly successful. If powdered casein and ovalbumen are immersed for some hours in M/5000 ferric chloride in 2/3 N.HCl (the solution used by Fischer and Hultzsch (1938)), the phosphoprotein casein gives a uniform pale blue stain with ammonium sulphide whereas ovalbumen is quite unstained. But when the tissue sections are treated in this way the amount of iron taken up by the nucleic acids is so small that staining is too faint to be of practical value. If the iron concentration is increased to M/2 ferric chloride in 2/3 N.HCl all the tissue constituents stain though rather faintly. Immersion in saturated iron alum followed by washing out in N/5 citric acid (pH 2·2), N/100 HCl (pH 2) or N/10 HCl (pH 1) gives tolerably good differentiation; the nucleic acid-containing structures in *Lumbricus* retain the iron and stain relatively more strongly. But it is clear that the curves of uptake or loss of iron by protein and nucleic acid in relation to pH are not sufficiently widely separated to give clear-cut differentiation by this means.

Metachromatic Staining with Iron

We have seen (p. 106) that iron sulphide gives a different tint in different tissues. This is most evident in sections of the scalp where the fibrous tissue stains chocolate brown, muscle grey-brown, nuclei blue-black, Malpighian layer blue-grey, stratum corneum and the keratohyalin granules of Huxley's layer pure blue, hair unstained. The same is to be seen in the developing 'egg tooth' of the chick, where all the cells of the future 'tooth' are filled with blue-black keratohyalin droplets and contrast sharply with the brown-staining cytoplasm of the cells beneath. In sections of *Lumbricus* most of the tissues stain varying shades of grey-brown, but the sperm heads stain blue-black, spermatids and other nuclei and nucleoli slate-grey, giant nerve-fibres and contents of mucous glands slate-grey.

Ammonium sulphide reduces ferric iron to ferrous sulphide. Since the sections are mostly blue or blue-grey in colour while immersed in ammonium sulphide, and become for the most part grey or grey-brown when washed in tap-water and exposed to the air, it seemed probable that this metachromatic staining results from a variable proportion of ferrous and ferric iron in the different tissues.

This has been tested by immersing the sections in iron alum and then treating with freshly prepared potassium ferrocyanide or ferricyanide. Neither of these salts alone will liberate iron from its combination with the tissues (p. 109), but in the presence of 1 per cent. hydrochloric acid some of the iron at least is set free and the tissues are stained blue. In general the amount of ferric iron present far exceeds that of ferrous: a much more intense blue staining is given by ferrocyanide. But the distribution of the colour after ferricyanide shows clearly that the blue, blue-black, or slate-grey quality in the iron sulphide stain is due to the presence of ferrous iron.

Thus in sections of the scalp treated with *ferricyanide*: the fibrous tissue is practically colourless except for the deep blue nuclei; the darkest blue struc

tures are the Malpighian layer, particularly the superficial horny layer, and the nuclei; the keratohyalin granules in the root sheaths are a deep clear blue, Henle's layer pale blue (fig. 3A); red blood corpuscles pale blue.

When the scalp sections are treated with *ferrocyanide*: the fibrous tissue is an intense blue; Malpighian layer pale blue, horny layer almost colourless; nuclei are darkly staining; keratohyalin granules glassy and refractile and quite colourless, Henle's layer colourless (fig. 3B); red blood corpuscles unstained.

FIG. 3. Sections of the root-sheath of hairs in the human scalp taken close to the bulb. A, iron alum followed by potassium ferricyanide in 1 per cent. HCl. B, iron alum followed by potassium ferrocyanide in 1 per cent. HCl. *a*, outer root-sheath; *b*, Henle's layer; *c*, Huxley's layer with granules of keratohyalin; *d*, hair with granules of pigment.

Similarly, in sections of *Lumbricus* all those structures which give a slate-grey tinge with iron sulphide stain blue with ferricyanide: nuclei and nucleoli, sperm heads, the core of the young spermatids, the giant nerve-fibres, and the networks in the giant nerve-cells. The muscles also stain blue and so do the contents of the mucous glands.

It is evident that certain of the cell structures, notably the nuclei, still contain reducing substances after fixation which prevent the oxidation of the ferrous sulphide to ferric. These substances possibly vary from one tissue to another. Golodetz and Unna (1909), who observed that the Malpighian layer of the skin would reduce potassium permanganate whereas the fibrous tissue would not, attributed this to the presence of tyrosine in keratin and its absence from collagen. But perhaps it is more probable that sulphydryl groups are responsible. Cystein will give a blue colour instantly in the cold with freshly mixed ferric chloride and potassium ferricyanide, whereas cystine will not, nor will tyrosine, glycine, glucose, or riboflavin. Di- and polyphenols (catechol, pyrogallol) likewise give immediate reduction; so does ascorbic acid; aldehydes (formaldehyde, acetaldehyde) do not.

It has been shown by Giroud and Bulliard (1930) that the soft keratin of the skin and the bulbs of the hair contains fixed —SH groups which give an intense nitroprusside reaction. The material responsible is regarded as the

precursor substance of keratin. When this is fully hardened, as in the hair itself, the sulphur is all in the oxidized -S—S- form and the nitroprusside reaction is negative. Collagen contains no cystine or cystein, which will explain the absence of ferrous iron in the fibrous tissue.

Whether the existence of sulphydryl groups in the side chains of the proteins will explain the presence of ferrous iron in the nuclei, muscles, &c., is less certain; but such groups are widely distributed in fixed tissues (Giroud, 1930) and may well be responsible. A number of proteins were tested by mixing with a drop of dilute potassium ferricyanide, leaving for 10 minutes, and then adding a drop of 0·1 per cent. ferric chloride in 1 per cent. nitric acid. Salmine and gelatine which contain no cystine give no blue colour; β-lactoglobulin gives a faint blue; ovalbumen, chromosomin (from calf thymus), and edestin a darker blue; histone from calf thymus, myosin, and tropomyosin the darkest blue.

The association of ferrous iron with nucleic acids in the cells is interesting. Neither ribonucleic acid nor desoxyribonucleic acid will reduce ferricyanide in the cold. Perhaps the associated histone or chromosomin are responsible, or perhaps some other unrecognized reducing substance is concerned.

Haematoxylin, Iron, and Aluminium

The differential staining of the tissues by haematoxylin is commonly attributed to differences in the affinity of the different structures for the iron-haematoxylin lake. The present observations suggest, however, that the differential affinity is for iron and the haematoxylin merely serves as an indicator for the varying quantities of iron taken up. Haematoxylin, like other mordant-fixed dyes, forms a very stable chelate compound or lake with iron (Werner, 1908; Race, Rowe, and Speakman, 1946) and further amounts of the dye are possibly added in the form of an adsorption complex (Weiser and Porter, 1928).

If a little haematoxylin (Heidenhain) is added to a solution of ferrous sulphate freshly prepared in boiled distilled water it gives a blue-violet colour; if it is added to a solution that has been kept for some days and contains an appreciable amount of ferric iron a purple colour is produced; with a solution of ferric ammonium sulphate it gives a chocolate-brown colour.

It was interesting, therefore. to examine the colours produced in a section of the scalp immersed in iron alum, placed for a brief period in Heidenhain's haematoxylin and then mounted without differentiation. All those structures (red blood corpuscles, stratum corneum, keratohyalin granules) which, as shown above, contain ferrous iron alone stain blue; structures (such as the Malpighian layer and the nuclei) which contain both ferrous and ferric iron stain purple; whereas the fibrous tissue, containing only ferric iron, stains brown.

The conclusions reached about the uptake of iron by the tissues probably apply equally to aluminium. Like iron, aluminium forms chelate complexes through oxygen (Sidgwick, 1950). In the mordanting of tissue sections it

probably competes for the same groups. Using ammonium sulphide as the indicator for iron, and alizarin as the indicator for aluminium, it can be shown that the relative intensity of staining with the two metals runs parallel. Moreover, the amount of iron taken up from 1 per cent. ferric chloride is reduced if the section has already been immersed in 10 per cent. aluminium sulphate, whereas treatment with iron depresses the subsequent uptake of aluminium.

There is a similar competition between iron and methylene blue (cf. Michaelis, 1947): the uptake of methylene blue by ovalbumen is almost completely inhibited by previous treatment with iron; but these matters have not been studied closely.

Conclusions

The simple and rapid methods for staining with the sulphides of iron and copper (p. 106) may prove useful for general histological purposes. The monochromatic sepia colouring given with copper is particularly well suited for photography. For low and medium-high powers the results are often equal to those given by iron haematoxylin, but at the highest magnifications they sometimes lack the sharpness and density of iron haematoxylin preparations.

The main interest of the observations lies, however, in the information which they give about the mechanism of the differential staining with compounds of iron. Such staining has the force of a rather crude histochemical test. According to the interpretation advanced in this paper it is a test for free acidic groups (free carboxyl and phosphoric acid groups); and the depth of staining is determined by (i) the relative abundance of such groups in the proteins and nucleic acids present and (ii) the concentration density of these substances in the tissues.

The observations on the staining of chromosomes with iron prove that this is often due as much to the proteins as to the nucleic acids. In some cases the protein seems to be the more important (p. 110) (cf. Stedman and Stedman, 1947).

Furthermore, the relative proportion of ferrous and ferric iron, which is revealed by the blueness or brownness of the colour of the iron sulphide or of the undifferentiated iron haematoxylin stain, is a measure of the concentration of reducing substances (chiefly sulphydryl groups) in the fixed tissues. High reducing power is characteristic of the nuclei and chromosomes, of the telophragma in striated muscle, of red blood corpuscles, and of keratohyalin and soft keratin. Complete absence of reducing power is characteristic of fibrous tissue.

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REFERENCES

BECHHOLD, H., 1928. Biochem. Z., **199**, 451.
BIRD, C. L., 1947. *The theory and practice of wool dyeing*, Bradford (Society of Dyers and Colourists).
FISCHER, F. G., and HULTZSCH, K., 1938. Biochem. Z., **299**, 104.
FRAENKEL-CONRAT, H., and OLCOTT, H. S., 1945. J. biol. Chem., **161**, 259.
GIERKE, H., 1884. Z. wiss. Mikr., **1**, 497.
GILSON, G., 1892*a*. Rep. Brit. Ass. Adv. Sci., 1892, 778.
—— 1892*b*. Ibid., 780.
GIROUD, A., 1930. C. R. Ass. Anat., **25**, 144.
——, and BULLIARD, H., 1930. Arch. Morph. gén. exp., **29**, 1.
GOLODETZ, L., and UNNA, P. G., 1909. Monatshefte f. prakt. Derm., **48**, 149.
KAUFMANN, B. P., GAY, H., and MCDONALD, M. R., 1950. Cold Spring Harbor Symp. quant. Biol., **14**, 85.
LANDOIS, L., 1865. Centralbl. med. Wiss., **3**, 867.
LEBER, T., 1868. Arch. Ophthalm., **14**, 300.
MACALLUM, A. B., 1908. Ergeb. Physiol., **7**, 552.
MICHAELIS, L., 1947. Cold Spring Harbor Symp. quant. Biol., **12**, 131.
POLICARD, A., 1934. Bull. Histol. appl., **11**, 216.
RACE, E., ROWE, F. M., and SPEAKMAN, J. B., 1946. J. Soc. Dyers and Colourists, **62**, 372.
SCHNEIDER, W. C., 1945. J. biol. Chem., **161**, 293.
SIDGWICK, N. V., 1950. *The chemical elements and their compounds*, Oxford.
SMYTHE, C. V., and SCHMIDT, C. L. A., 1930. J. biol. Chem., **88**, 241.
STEDMAN, E., and STEDMAN, E., 1947. Symp. Soc. exp. Biol., **1**, 232.
TOMPSETT, S. L., 1934. Biochem. J., **28**, 1802.
TRISTRAM, G. R., 1949. Advances in Protein Chemistry, **5**, 83.
WEISER, H. B., and PORTER, E. E., 1928. Colloid Symp. Monograph, **5**, 369.
WERNER, A., 1908. Ber. deutsch. chem. Gesell., **41**, 1062.
WIENER, A., 1916. Biochem. Z., **77**, 27.

BRAND **MICHROME** BRAND

STAINS & REAGENTS

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Acridine Yellow
Adonitol
Auramine, O
Azo Carmine, B & G
Azo Eosin
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